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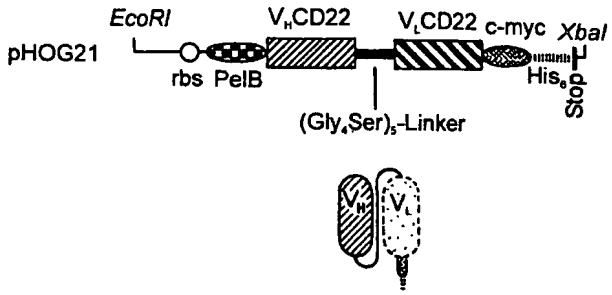
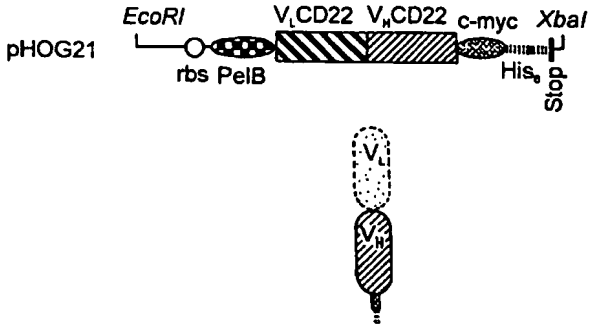
(54) Title: NOVEL STABLE ANTI-CD22 ANTIBODIES

(57) Abstract: The present invention provides stable anti-CD22 antibodies, nucleic acids encoding stable anti-CD22 antibodies, and therapeutic and diagnostic methods and compositions using stable anti-CD22 antibodies.

(A) scFv with 15 amino acid GlySer-linker in V_H-V_L orientation



(B) scFv without linker (zero-linker construct) in V_L-V_H orientation



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Novel Stable Anti-CD22 Antibodies

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. provisional application no. 60/387,306, filed June 7, 2003, which is herein incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] Monoclonal antibodies provide powerful diagnostic and therapeutic tools for fighting malignant disease. Recombinant antibody technology has provided the basis to isolate gene segments encoding only the variable antigen binding domains of monoclonal antibodies. Constructs encoding only the variable antigen binding domains can be expressed, for example in *E. coli*. Upon expression, variable antigen binding domains retain their antigen binding properties. Variable antibody domains of the light chain (V_L) and heavy chain (V_H) can be stabilized by the introduction of a synthetic linker peptide, resulting in a single chain Fv molecule (scFv). In contrast to "intact" antibodies, scFv are able to penetrate tumor tissue efficiently due to their small size. This property makes scFv antibodies ideal candidates for the construction of cytotoxic fusion proteins. Since the immunogenicity of an antibody is primarily mediated by its constant domains, scFv antibodies also have a very low immunogenic potential and are therefore well suited as novel immunotherapeutics.

[0003] Multivalent antibody fragments based on the scFv format are also of particular interest for several reasons. The simultaneous binding to two or more target antigens leads to an increase in functional affinity due to the avidity effect. *See, e.g.*, Crothers and Metzger (1972) *Immunochem.* 9(3):341. Consequently, multivalency has a significant influence on the dissociation kinetics, which is of particular importance under non-equilibrium conditions of antibody-antigen interactions. Further more, there is evidence that receptor mediated endocytosis can be enhanced by antibodies or antibody fragments capable of receptor dimerization. *See, e.g.*, Becerril *et al.* (1999) *Biochem, Biophys. Res. Commun.* 255(2):386, Heldin (1995) *Cell* 80(2):213, and Yarden (1990) *Proc. Natl. Acad. Sci. USA* 94(18):9637.

[0004] It has also been shown that some homodimers of monoclonal antibodies have antitumor activity, *i.e.*, by signaling target cells to undergo cell cycle arrest or apoptosis. *See, e.g.*, Ghetie *et al.* (2001) *Blood* 97(5):1392 and Ghetie *et al.* (1997) *Proc. Natl. Acad. Sci.*

USA 94(14):7509. Engineering scFv antibody fragments for multivalency also increases their size which yields more favorable constructs with respect to biodistribution and pharmacokinetics.

[0005] One approach to increasing the valency of scFv fragments has been to shorten the length of the linker connecting the variable domains. Reducing the linker length to 3-12 residues prevents pairing between domains of the same polypeptide chain and forces pairing between complementary domains of a second scFv molecule to form a bivalent diabody. *See, e.g.*, Hollinger *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444. When the linker is further shorted to less than 3 residues, the variable domains can associate for form trivalent "triabodies" or tetravalent "tetrabodies". *See, e.g.*, Atwell *et al.* (1999) *Protein Eng.* 12(7):597, Dolezal *et al.* (2000) *Protein Eng.* 13(8):565, Kortt *et al.* (1997) *Protein Eng.* 10(4):423, Le Gall *et al.* (1999) *FEBS Lett.* 453(102):164, Pei *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94(18):9637. Manipulation of linker length therefore provides a convenient method for modulating the valency and therefore the functional affinity of scFv antibodies.

[0006] The CD22 antigen is strongly overexpressed in the majority of B cell non-Hodgkin lymphomas. Thus, the antigen represents a good target for antibody based cytotoxic fusion proteins. Many attempts have been made to generate a stable anti-CD22-scFv antibody derived from the clinically established monoclonal antibody LL2. These endeavors, however, have failed. To date, no stable single chain LL2 antibody has been developed.

[0007] Thus, there is a need in the art to develop stable single chain antibodies. This invention addresses that need and provides stable anti-CD22 antibodies for use in therapeutic and diagnostic applications.

BRIEF SUMMARY OF THE INVENTION

[0008] The present invention provides stable anti-CD22 antibodies, nucleic acids encoding the antibodies, and therapeutic and diagnostic methods and compositions using the antibodies.

[0009] Thus, the invention provides a stable anti-CD22 antibody having a V_H domain and a V_L domain, wherein the sequence of the V_H and V_L domains is at least 80%, often 85%, 90%, or 95%, 98%, or 99%, identical to the V_H and V_L domain sequences set forth in SEQ ID NO:1 (amino acid residues at positions 1-116 of SEQ ID NO:1 and amino acid residues at positions 132-244 of SEQ ID NO:1, respectively); and has at least one of the following amino

acid substitutions: Gln replacing Glu at position 6 of the V_H domain, Val replacing Ser at position 12 of the V_H domain, or Lys replacing Asn at position 62 of the V_H domain. Typically, the antibody comprises at least two of the substitutions. For example, in some embodiments, position 6 of the V_H domain is a Gln and position 12 is a Val. In other embodiments, position 6 of the V_H domain is a Gln and position 62 is a Lys. Often, the antibody comprises all three of the substitutions. Often, the antibody comprises a V_L domain sequence as set forth in SEQ ID NO:1 and a V_H domain sequence as set forth in SEQ ID NO:1 with the indicated substitutions.

.**[0010]** In some embodiments, the V_H domain and the V_L domains are separated by a linker. In other embodiments, they are adjacent. The V_H and V_L domains may be in either orientation, but in some embodiments, are in a V_L - V_H orientation.

[0011] The antibody can further comprises additional substitutions. For example, in one embodiment, the amino acid at position 6 of the V_H domain is Gln, the amino acid at position 12 of the V_H domain is Val, the amino acid at position 62 of the V_H domain is Lys, and the amino acid at position 25 of the V_L domain is Ala. Such a variant antibody can also comprise additional substitutions. For instance, in one embodiment, an antibody of the invention comprises a Gln at position 6 of the V_H domain, a Val at position 12 of the V_H domain, a Lys at position 62 of the V_H domain, an Ala at postion 25 of the V_H domain, and a Met at position 4 of the V_L domain.

[0012] In another aspect, the invention provides a stable humanized anti-CD22 antibody having an LL2 binding specificity where the sequence of the V_H and V domains is at least 70% identical to SEQ ID NO:1, and the antibody has at least one, typically two, of the following amino acid substitutions: Gln replacing Glu at position 6 of the V_H domain; Val replacing Ser at position 12 of the V_H domain; and Lys replacing Asn at position 62 of the V_H domain. The stable humanized anti-CD22 antibody may further comprise additional amino acid substitutions.

[0013] In another aspect, the invention provides a nucleic acid encoding a stable anti-CD22 antibody of any of the embodiments set forth above and described herein.

[0014] In an additional aspect, the invention provides an immunoconjugate comprising a stable anti-CD22 antibody of any of the embodiments set forth above. In some embodiments, the immunoconjugate is recombinantly produced. The immunoconjugate may comprise an antibody linked to an effector molecule, which can be a therapeutic moiety, for example, a

small molecule, or drug, an enzyme, a cytokine, or a toxin. In some embodiments, the enzyme is an RNAse, *e.g.*, angiogenin or RapLR1. In particular embodiments, the cytokine is IL-2, IL-6, or IL-12.

[0015] In another aspect, the invention provides a nucleic acid encoding an immunoconjugate as set forth above.

[0016] In another aspect, the invention provides, a method of inhibiting growth of a malignant B cell, said method comprising: contacting the cell with a composition comprising a stable anti-CD22 antibody of the invention. For example, such a composition can be the antibody linked to a toxic moiety. The cell may be a mammalian cell, for example a human cell. In particular embodiments, the malignant B cell is from a lymphoma, for example B cell non-Hodgkin's lymphoma.

[0017] In another aspect, the invention provides a method of detecting a cell that expresses CD22, said method comprising the steps of: contacting the cell with a composition comprising an antibody as described above; and detecting binding of the antibody to the cell. The cell may be a mammalian cell, *e.g.*, a human cell. Such a method may be performed *in vitro* or *in vivo*. In some embodiments, *e.g.*, use *in vivo*, the method may be therapeutic. In other embodiments, *e.g., in vitro* uses, the method may be diagnostic.

[0018] In another aspect the invention provides a stable anti-CD22 antibody, or a nucleic acid encoding the antibody, having a V_H domain and a V_L domain, wherein the sequence of the V_H and V_L domain is at least 90% identical to SEQ ID NO:1 and having at least one of the following amino acid substitutions: a Gln or a conservative substitution thereof at position 6 of the V_H domain; a Val or a conservative substitution thereof at position 12 of the V_H domain; or a Lys or a conservative substitution thereof at position 62 of the V_H domain.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1 depicts a schematic representation of anti-CD22 single chain fragments. Figure 1A depicts scFv with a 15 amino acid GlySer linker in the V_H-V_L orientation. Figure 1B depicts scFv without linkers in the V_L-V_H orientation.

[0020] Figure 2A and Figure 2B illustrated binding of single chain LL2 antibody variants (340 nM) to $CD22^+$ Raji cells by flow cytometry. Figure 2A illustrates flow cytometric analysis of the unstable wildtype single chain antibody MLZ1.1 and a stable antibody,

MLV5.1. Figure 2B shows the median value of the fluorescence intensity shift is shown for each single chain LL2 antibody variant.

[0021] Figure 3A and 3B illustrates the serum stability of wild type single chain LL2 antibody in comparison with single chain LL2 antibody variants. Half-life of the single chain LL2 antibodies in human serum at 37°C was measured. Immunoreactivity with $CD22^+$ Raji cells was assayed by flow cytometry after incubating the constructs at 37°C in human serum for various time points. The half-life was determined as the time point with 50% of the initial binding activity.

[0022] Figure 4 shows the purification and serum stability of engineered scFV MJ-7. (A) is a western blot analysis of purified MJ-7. Size of molecular weight markers are indicated (kDa). (B) shows the immunoreactivity of monomeric MJ-7 (diamonds) and MLZ-wt (squares) after incubation in human serum at 37°C over a period of six days.

[0023] Figure 5 illustrates the affinity and specificity of wild-type scFv and stability engineered scFv. (A) Equilibrium-binding curves for mAb LL2 (open circles); MLZ-wt (closed squares) and MJ-7 (closed triangles) as determined by flow cytometry. Binding activity to Raji cells at indicated concentrations is shown as median fluorescence intensity (MFI) minus background fluorescence. Measurements were performed in triplicate; standard deviations are shown as bars. Binding affinity constants (K_D) were determined by fitting the cell binding data to the nonlinear regression model according to the Levenberg-Marquard method. (B) Epitope specificity was determined by competition experiments and analyzed by flow cytometry. MLZ-wt (closed squares) and MJ-7 (closed triangles) competed with the mAb LL2 for binding to the CD22 antigen on Raji cells. Results are shown as percent binding inhibition of the mAb when incubating tumor cells with various concentrations of scFvs.

[0024] Figure 6 illustrates flow cytometric analysis of the V_L-V_H zero linker construct, a stable single chain LL2 antibody variant MLV5.1, and the monoclonal antibody LL2.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0025] The present invention provides improved, more stable anti-CD22 antibodies and nucleic acids that encode them. The present invention is based on the discovery that particular positions within the V_H and V_L domains of the LL2 antibody are important for

stability of the antibody. The improved anti-CD22 antibodies are novel variants of the anti-CD22 monoclonal antibody LL2, which is described in Leung *et al.* (1994) *Hybridoma* 13(6):469. These antibodies have increased stability in comparison to a single chain antibody comprising the wild type LL2 V_H and V_L sequences. In some embodiments, the antibodies comprise a linker between the V_H and V_L domains (*see, e.g,* Figure 1A). In one embodiment, a zero linker variant of a single chain LL2 antibody produces a homogeneous antibody preparation corresponding to a stable dimeric single chain LL2 antibody (Figure 1B).

[0026] The invention also provides immunoconjugates comprising the novel anti-CD22 antibodies and methods of using the antibodies and immunoconjugates in therapeutic and diagnostic applications that target CD22-bearing cells.

II. Definitions

[0027] As used herein, the following terms have the meanings ascribed to them below unless otherwise specified.

[0028] The term "antibody" refers to a polypeptide encoded by an immunoglobulin gene or functional fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0029] An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

[0030] Examples of antibody functional fragments include, but are not limited to, complete antibody molecules, antibody fragments, such as Fv, single chain Fv (scFv), complementarity determining regions (CDRs), V_L (light chain variable region), V_H (heavy chain variable region), Fab, F(ab)2' and any combination of those or any other functional portion of an immunoglobulin peptide capable of binding to target antigen (*see, e.g.*, Fundamental Immunology (Paul ed., 3d ed. 1993). As appreciated by one of skill in the art, various

antibody fragments can be obtained by a variety of methods, for example, digestion of an intact antibody with an enzyme, such as pepsin; or *de novo* synthesis. Antibody fragments are often synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies (*e.g.*, single chain Fv) or those identified using phage display libraries (*see, e.g.*, McCafferty *et al.*, (1990) Nature 348:552). The term antibody also includes bivalent or bispecific molecules, diabodies, triabodies, and tetrabodies. Bivalent and bispecific molecules are described in, *e.g.*, Kostelny *et al.*. (1992) *J. Immunol.* 148:1547, Pack and Pluckthun (1992) *Biochemistry* 31:1579, Hollinger *et al.*(1993), *supra*, Gruber *et al.* (1994) *J Immunol.* :5368, Zhu *et al.* (1997) *Protein Sci.* 6:781, Hu *et al.* (1996) *Cancer Res.* 56:3055, Adams *et al.* (1993) *Cancer Res.* 53:4026, and McCartney, *et al.* (1995) *Protein Eng.* 8:301.

[0031] References to "V_H" or a "VH" refer to the variable region of an immunoglobulin heavy chain, including an Fv, scFv , dsFv or Fab. References to "V_L" or a "VL" refer to the variable region of an immunoglobulin light chain, including of an Fv, scFv , dsFv or Fab.

[0032] As used herein, a "stable anti-CD22 antibody" or "anti-CD22 antibody variant" or "stable LL2 antibody" or "stable LL2 variant antibody" refers to an antibody comprising an anti-CD22 LL2 V_H joined to an anti-CD22 LL2 V_L region, in which at least one of the variable regions comprises one or more substitutions set forth herein. Thus, a "stable anti-CD22 antibody" may have a V_H and V_L region as set forth in SEQ ID NO:1 in which specific residues in the variable regions are substituted at particular positions, disclosed herein, to enhance stability. Amino acid residues at positions 1-116 of SEQ ID NO:1 correspond to a V_H region and amino acid residues at positions 132-244 of SEQ ID NO:1 correspond to a V_L region. (The amino acid residues at positions 117-131 of SEQ ID NO:1 correspond to a peptide linker.) The term "stable anti-CD22 antibody" or "anti-CD22 antibody variant" encompasses embodiments such as single chain antibodies, including diabodies and other multivalent antibody constructs. The variable regions may be joined in either orientation, either directly or through a linker. For example, a "stable anti-CD22 antibody" or "anti-CD22 antibody variant" may be a stable LL2 antibody or LL2 antibody variant. As understood by one of skill in the art, a stable antibody of the invention retains the binding specificity of the parent LL2 antibody. Thus, the regions of the CDRs that determine binding specificity are typically unchanged. A stable anti-CD22 antibody of the invention has a half

life in human serum at 37° that is at least 2 hours, typically at least 5 or 10 hours, and most often greater than 20 or 50 hours.

[0033] The positions of amino acid residues in an antibody heavy chain or light chain are determined herein in accordance with standard numbering as set forth in Kabat *et al.*, (1991) *Sequences of Proteins of Immunological Interest* 5th Ed., NIH Publication No. 91-3242. *See also*, Johnson and Wu (2001) *Nuc. Acids Res.* 29:205. Therefore, when making a comparison between an amino acid sequence and SEQ ID NO:1, the two sequences are aligned for maximal correspondence over a comparison window, or designated region, as described below. For example, the amino acid at position 6 of the V_H domain may serve as a reference point. Upon maximal alignment of an amino acid sequence encoding another V_H domain, the corresponding position may be determined by comparison. The residue is considered to be at position 6 even though, it may not be residue number 6 in the V_H sequence.

[0034] The term "binding specificity," "specifically binds to an antibody" or "specifically immunoreactive with," when referring to a CD22 epitope refers to a binding reaction which is determinative of the presence of the CD22 epitope in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular CD22 epitope at least two times the background and more typically more than 10 to 100 times background. In particular, an LL2 variant antibody of the invention retains the binding specificity of the parent LL2 antibody. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein or carbohydrate. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein or carbohydrate. *See, e.g.*, Harlow & Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

[0035] An anti-CD22 or LL2 antibody of the present invention may be a "humanized" antibody. A "humanized anti-CD22 or LL2 antibody" refers to an antibody in which the antigen binding loops, *i.e.*, CDRs, comprised by the V_H and V_L regions are grafted to a human framework sequence. In some embodiments, a "humanized anti-CD22 antibody" of the invention comprises V_H and V_L regions in which a substitution resulting in enhanced

stability is present in at least one position selected from the group consisting of L25, H12, or H62. Often, substitutions are present in each of these positions.

[0036] A stable anti-CD22 or LL2 antibody of the present invention may be a portion of an "immunoconjugate." The term "immunoconjugate" refers to a stable anti-CD22 antibody of the invention linked to an "effector molecule." "Effector molecule" refers to the portion of an immunoconjugate intended to have a function other than targeting of the conjugate to a cell or molecule of interest. As used herein, an "effector molecule" is a detectable label or a therapeutic moiety.

[0037] A "therapeutic moiety" is the portion of an immunoconjugate intended to act as a therapeutic agent.

[0038] The term "therapeutic agent" includes any number of compounds currently known or later developed to act as anti-neoplastics, anti-inflammatories, anti-infectives, enzyme activators or inhibitors, allosteric modifiers, antibiotics or other agents administered to induce a desired therapeutic effect in a patient. The therapeutic agent may also be a toxin or a radioisotope, where the therapeutic effect intended is, for example, the killing of a cancer cell.

[0039] In the context of an immunoconjugate, a "detectable label" refers to, a portion of the immunoconjugate which has a property rendering its presence detectable. For example, the immunoconjugate may be labeled with a radioactive isotope which permits cells in which the immunoconjugate is present to be detected in immunohistochemical assays. A "detectable label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include radioisotopes (*e.g.*, ^{3}H, ^{35}S, ^{32}P, ^{51}Cr, or ^{125}I), fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, alkaline phosphatase, horseradish peroxidase, or others commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, e.g., by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

[0040] The terms "effective amount" or "amount effective to" or "therapeutically effective amount" refers to an amount sufficient to induce a detectable therapeutic response in the subject. Preferably, the therapeutic response is effective in reducing the proliferation of cancer cells or in inhibiting the growth of cancer cells present in a subject. Assays for determining therapeutic responses are well known in the art.

[0041] The term "linked" in the context of the immunoconjugates of the present invention refers to the linkage between the stable anti-CD22 or LL2 antibody of the present invention and the effector molecule. The linkage may be introduced through recombinant means or chemical means. Methods of introducing linkages recombinantly are well known to those of skill in the art and are described below. Exemplary chemical linkages include, for example, covalent bonding, including disulfide bonding; hydrogen bonding; electrostatic bonding; recombinant fusion; and conformational bonding, *e.g.*, antibody-antigen, and biotin-avidin associations. Additional linkers and methods of linking are described in WO 98/41641.

[0042] The term "single chain antibody" typically refers to an antibody wherein the genetic information encoding the functional fragments of the antibody are located in a single contiguous length of DNA. Single chain antibodies are described *e.g.*, in Bird, *et al.*, (1988) *Science* 242:423 and Huston, *et al.*, (1988) *Proc. Natl. Acad. Sci. USA* 85:5879.

[0043] The term "surface marker" refers to a protein, carbohydrate, or glycoprotein present on the surface of a cell. Different types of cells express different cell surface markers and therefore cells can be identified by the presence of a cell surface marker. For example, malignant B cells overexpress CD22.

[0044] "Immunoassay" refers to a method of detecting an analyte in a sample in which specificity for the analyte is conferred by the specific binding between an antibody and a ligand. This includes detecting an antibody analyte through specific binding between the antibody and a ligand. Multiple immunoassay formats and conditions are known in the art and are described in *e.g.*, Harlow and Lane (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York.

[0045] "Nucleic acid" and "polynucleotide" are used interchangeably herein to refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

[0046] "Codon" refers to a nucleotide sequence that specifies an amino acid or represents a signal to initiate or stop a function. Unless otherwise indicated, a particular nucleic acid sequence also encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[0047] "Polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to naturally occurring amino acid polymers, as well as, amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid.

[0048] "Amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. "Amino acid analogs" refers to compounds that have the same fundamental chemical structure as a naturally occurring amino acid, *i.e.*, an alpha carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. "Amino acid mimetics" refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

[0049] "Conservatively modified variants" applies to both nucleic acid and amino acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino

acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0050] With respect to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologues, and alleles of the invention.

[0051] For example, substitutions may be made wherein an aliphatic amino acid (G, A, I, L, or V) is substituted with another member of the group. Similarly, an aliphatic polar-uncharged group such as C, S, T, M, N, or Q, may be substituted with another member of the group; and basic residues, *e.g.*, K, R, or H, may be substituted for one another. In some embodiments, an amino acid with an acidic side chain, E or D, may be substituted with its uncharged counterpart, Q or N, respectively; or vice versa. Each of the following eight groups contains other exemplary amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);

7) Serine (S), Threonine (T); and

8) Cysteine (C), Methionine (M)

(see, *e.g.*, Creighton, Proteins (1984)).

[0052] Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, see, *e.g.*, Alberts *et al.*, *Molecular Biology of the Cell* (3rd ed., 1994) and Cantor and Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 50 to 350 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of β-sheet and α-helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units.

[0053] The terms "isolated" or "substantially purified," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state, although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified.

[0054] The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure. The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

[0055] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region (e.g., amino acid sequence SEQ ID NO: 1 or 2), when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection. Such sequences are then said to be "substantially identical." This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

[0056] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0057] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman (1981) *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson & Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575

Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[0058] A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al., supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For the purposes of this application, percent identity is determined using the default setting of BLAST.

[0059] The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic

acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

[0060] "Promoter" and "expression control sequence" are used herein to refer to an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

[0061] An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

[0062] The term "nucleic acid encoding" or "nucleic acid sequence encoding" refers to a nucleic acid which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The nucleic acid sequences include both full length nucleic acid sequences as well as shorter sequences derived from the full length sequences. It is understood that a particular nucleic acid sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. The nucleic acid includes both the sense and antisense strands as either individual single strands or in the duplex form.

[0063] The term "pharmaceutical composition" refers to formulations of various preparations. Parenteral formulations are known and are preferred for use in the invention.

The formulations containing therapeutically effective amounts of the immunoconjugates are either sterile liquid solutions, liquid suspensions or lyophilized versions and optionally contain stabilizers or excipients. Lyophilized compositions are reconstituted with suitable diluents, *e.g.*, water for injection, saline, 0.3% glycine and the like, at a level of about from 0.01 mg/kg of host body weight to 10 mg/kg or more.

III. Stabilized Anti-CD22 Antibodies

[0064] The stable anti-CD22 antibodies described herein were generated as variants of a wild type single chain LL2 antibody (see, *e.g.*, Leung *et al, supra*). Generation of the anti-CD22 antibodies is described in Example 1 below. A stable anti-CD22 antibody of the invention comprises a LL2 V_H and a LL2 V_L region in which at least one of the variable regions has one or more substitutions that result in enhanced stability. The improved antibodies of the invention contain at least one of the following substitutions relative to the wildtype sequence provide in SEQ ID NO:1: a Gln replacing Glu at position 6 of the V_H domain, a Val replacing Ser at position 12 of the V_H domain, a Lys replacing Asn at position 62 of the V_H domain. Typically, the improved antibodies contain at least two of the substituted residues. For example, in some embodiments, the antibodies of the invention comprise a Gln at position 6 of the V_H domain and one of the other two substitutions. Most often, an improved antibody comprises all three of the substitutions and therefore has a Gln at position 6 of the V_H domain, a Val at position 12 of the V_H domain and a Lys and position 62 of the V_H domain.

[0065] As understood by one of skill in the art, the antibody variants of the invention may also comprise other substitutions, *e.g.*, conservative changes, that do not substantially alter antibody binding specificity or stability, *i.e.*, the conservatively substituted variant retains improved stability relative to the wild-type parent sequence. Such a conservative substitution can often be made in a region that is outside of the central core of the variable domains. The central core region can be determined as described by Chothia *et al., J. Mol. Biol.* 278:457-479, 1998). For example, a variant with an additional conservative substitution can comprise a substitution where an Ala residue replaces the Ser residue at position 25 of the V_L domain as set forth in SEQ ID NO:1. The effects of such substitutions on stability can be easily tested as described herein.

[0066] Stability refers to thermal stability, *e.g.*, stability in serum at 37°C, and resistance to degradation, *e.g.*, resistance to proteolysis in serum at 37°C. Enhanced stability is determined by comparing a single chain anti-CD22 antibody comprising the variant, *i.e.*, substituted, V_H and/or V_L region to, for example, a single chain LL2 antibody comprising the wild type LL2 V_H and V_L regions. The thermal stability and resistance to proteolysis of the anti-CD22 antibodies can be assayed, for example, as set forth in Example 2. An anti-CD22 antibody of the invention typically has at least 5-fold more stability in serum compared to the wild type anti-CD22 antibody, often 10-fold, 20-fold, 25-fold, 50-fold, or 100-fold more stability.

[0067] The positions for substitution are determined with reference to the V_H and V_L sequences as set forth in SEQ ID NO:1. It should be understood that such position designations do not indicate the number of amino acids in the claimed molecule per se, but indicate where in the claimed LL2 antibody the residue occurs when the amino acid sequences of the V_H and V_L regions of the claimed antibody are maximally aligned with the V_H and V_L sequences of SEQ ID NO:1. Alignment can be performed either manually or using a sequence comparison algorithm described above.

[0068] An antibody variant of the invention binds to the same epitope as the parent anti-CD22 antibody. Methods to determine whether the antibody binds to the same epitope are well known in the art, *see, e.g.*, Harlow & Lane, Using Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1999, which discloses techniques to epitope mapping or alternatively, competition experiments, to determine whether an antibody binds to the same epitope as the donor antibody. A stable anti-CD22 antibody variant of the invention may exhibit altered affinity when compared to a single chain antibody comprising the wild type variable regions. The affinity of the single chain anti-CD22 variant for CD22, may, for example, be decreased compared to the wild type anti-CD22 single chain antibody. Such a decrease may be by as much as 10-fold in comparison, but typically an anti-CD22 variant of the invention has an affinity that is at least 25%, more often at least 50% of that of a wild type anti-CD22 antibody. In some embodiments, the affinity for CD22 is increased, such that an anti-CD22 antibody of the invention has an affinity that is 2 times and sometime 5, 10, 50, or 100 times the affinity of the wild type anti-CD22 antibody. Affinity may be tested as set forth in Example 4.

[0069] Stable anti-CD22 antibodies of the present invention may be generated using routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods

used in the present invention include Sambrook *et al., Molecular Cloning, A Laboratory Manual* (3d ed. 2001); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al., eds.*, 1994)). For example, the

[0070] One embodiment of the present invention provides nucleic acid constructs that encode single chain anti-CD22 antibodies described here. A nucleic acid construct is one which, when incorporated into an appropriate vector, is capable of replicating in a host. The constructs may be linked to other sequences capable of affecting the expression of the construct, such as promoters and enhancers.

[0071] The V_H and V_L domains of the anti-CD22 antibody may be directly linked or may be separated by a linker, *e.g.* to stabilize the variable antibody domains of the light chain and heavy chain, respectively. Suitable linkers are well known to those of skill in the art and include the well known GlyGlyGlyGlySer linker or a variant thereof. Often the amino acid sequence of the linker is: GGGGSGGGGSGGGGS (SEQ ID NO: 26) encoded by a nucleotide sequence such as: 5' GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGG TGGCGGATCA 3' (SEQ ID NO:25). Other linkers, including hinge regions, that can be used in the invention include those described, for example in Alfthan *et al, Protein Eng.* 8(7), 725-31; Choi *et al, Eur. J. Immunol.* 31(1), 94-106; Hu *et al, Cancer Res.* 56(13), 3055-61; Kipriyanov, *et al, Protein Eng.* 10(4), 445-53; Pack, *et al, Biotechnology* (N Y) 11(11), 1271-7; and Roovers, *et al, Cancer Immunol. Immunother.* 50(1):51-9.

[0072] The V_L and V_H domains of the stable anti-CD22 antibodies disclosed herein may be linked in either a V_L-V_H orientation or a V_H-V_L orientation. Anti-CD22 antibody structural units may form one component of a "reconstituted" antibody or antibody fragment, *e.g.*, a Fab, a Fab' monomer, a F(ab)'2 dimer, or an whole immunoglobulin molecule. Other exemplary antibody structural units comprise the V_H or V_L chains alone. The stable anti-CD22 antibody of the present invention may further comprise a Fc portion. Even other exemplary stable anti-CD22 antibody structural units comprise multivalent forms of single chain anti-CD22 antibodies such as, for example, bivalent or bispecific molecules, diabodies, triabodies, and tetrabodies. Bivalent and bispecific molecules are described in, *e.g.*, Kostelny *et al.* (1992) *J. Immunol.* 148:1547, Pack and Pluckthun (1992) *Biochemistry* 31:1579, Hollinger *et al.*, 1993, *supra*, Gruber *et al.* (1994) *J. Immunol.* :5368, Zhu *et al.* (1997) *Protein Sci* 6:781, Hu *et al.* (1996) *Cancer Res.* 56:3055, Adams *et al.* (1993) *Cancer Res.*

53:4026, and McCartney, *et al.* (1995) *Protein Eng.* 8:301. Bispecific antibody fragments comprising an anti-CD22 antibody of the present invention may be used, for example to target B cells, cytotoxic T cells, or natural killer cells. *See, e.g.*, Holliger *et al.* (1996) *Prot. Eng.* 9:299, Zhu *et al.* (1996) *Biotechnology* 14:192, Kipriyanov *et al* (1998) *Int. J. Cancer* 77:763, Manzke *et al* (1999) *Int. J. Cancer* 82:700, Arndt *et al.* (1999) *Blood* 94:2562. Multivalent antibodies are described, *e.g.*, in Coloma and Morrison (1997) *Nat Biotechnol* 15:159, Alt *et al.* (1999) *FEBS Lett* 454:90, and Kipriyanov *et al* (1999) *J. Mol. Biol.* 293:41.

[0073] In some embodiments, an anti-CD22 antibody of the invention is a "humanized" antibody. Humanized antibodies typically comprise the wild type antibody CDRs within a human framework. Humanized antibodies have 70, 75, 80, 85% or greater identity to the framework regions of the V_H and V_L regions of SEQ ID NO:1. The particular positions in the stable anti-CD22 antibodies identified herein correspond to positions in the humanized antibody as determined by the Kabat numbering system (*see, e.g.*, Kabat *et al.* (1991) *supra*). Typically, the humanized antibodies have the same anti-CD22 binding specificity as the stable anti-CD22 antibodies described herein. Techniques for humanizing antibodies are well known in the art and are described in *e.g.*, U.S. Patent Nos. 4,816,567; 5,530,101; 5,859,205; 5,585,089; 5,693,761; 5,693,762; 5,777,085; 6,180,370; 6,210,671; and 6,329,511; WO 87/02671; EP Patent Application 0173494; Jones *et al.* (1986) *Nature* 321:522; and Verhoyen *et al.* (1988) *Science* 239:1534. Humanized antibodies are further described in, *e.g.*, Winter and Milstein (1991) *Nature* 349:293. Humanized LL2 antibodies are described in, *e.g.*, Leung, *et al.*, (1995) *Mol. Immunol.* 32:1413; and U.S. Patent Nos. 5,789,554, 6,187,287, and 6,254,868.

[0074] Oligonucleotides for use in generating nucleic acids encoding the antibodies described herein that are not commercially available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers (1981) *Tetrahedron Letts.* 22:1859, using an automated synthesizer, as described in Van Devanter *et. al.*, (1984) *Nucleic Acids Res.* 12:6159. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in, *e.g.*, Pearson & Reanier, (1983) *J. Chrom.* 255:137.

[0075] The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, (1981) *Gene* 16:21.

[0076] Typically, the nucleic acid sequences encoding anti-CD22 antibodies are generated using overlap extension PCR as described in Example 1. Overlapping primers that correspond to the V_H and V_L domains of an anti-CD22 antibody may be designed based on the sequence of an anti-CD22 antibody (*see, e.g.*, Leung, *et al.* (1994) *supra*).

[0077] The nucleic acid sequences encoding anti-CD22 antibodies and related nucleic acid sequence homologues may also be cloned from cDNA from the hybridoma lines producing anti-CD22 antibodies. For example, nucleic acid sequences encoding anti-CD22 antibodies may be cloned from the hybridoma line, EPB-2, *e.g.*, poly(A)$^+$ RNA extracted from EPB-2 hybridoma cells is reverse transcribed using appropriate primers. The V_H and V_L domains are amplified separately by two polymerase chain reactions (PCR). Nucleic acid sequences encoding anti-CD22 antibodies and related nucleic acid sequence homologues may also be cloned from cDNA or genomic DNA libraries or isolated using amplification techniques with oligonucleotide primers. One of skill in the art will recognize multiple suitable primers may be used.

[0078] Mutations in the V_H and V_L domains may be introduced using a number of methods known in the art. These include site-directed mutagenesis strategies such as overlap extension PCR (*see, e.g.*, Sambrook & Russell, *supra*; Ausubel *et al., supra*). Exemplary techniques and primers are provided in Examples 1.

[0079] The PCR products are subcloned into suitable cloning vectors that are well known to those of skill in the art and commercially available. Clones containing the correct size DNA insert are identified, for example, agarose gel electrophoresis. The nucleotide sequence of the heavy or light chain coding regions is then determined from double stranded plasmid DNA using the sequencing primers adjacent to the cloning site. Commercially available kits (*e.g.*, the Sequenase® kit, United States Biochemical Corp., Cleveland, OH) are used to facilitate sequencing the DNA.

[0080] One of skill will appreciate that, utilizing the sequence information provided for the variable regions, nucleic acids encoding these sequences are obtained using a number of methods well known to those of skill in the art. Thus, DNA encoding the variable regions is prepared by any suitable method, including, for example, amplification techniques such as ligase chain reaction (LCR) (*see, e.g.*, Wu & Wallace (1989) *Genomics* 4:560, Landegren, *et al.* (1988) *Science* 241:1077, and Barringer, *et al.* (1990) *Gene* 89:117), transcription amplification (*see, e.g.*, Kwoh, *et al.* (1989) *Proc. Natl Acad. Sci. USA* 86:1173), and self-

sustained sequence replication (*see, e.g.*, Guatelli, *et al.* (1990) *Proc. Natl Acad. Sci. USA* 87:1874), cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang, *et al.*, (1979) *Meth. Enzymol.* 68:90; the phosphodiester method of Brown, *et al.*, (1979) *Meth. Enzymol.* 68:109; the diethylphosphoramidite method of Beaucage, *et al.*, (1981) *Tetra. Lett.* 22:1859; and the solid support method of U.S. Patent No. 4,458,066.

[0081] The nucleic acid sequences that encode the single chain antibodies, or variable domains, are identified by techniques well known in the art (*see*, Sambrook, *et al., supra*). Briefly, the DNA products described above are separated on an electrophoretic gel. The contents of the gel are transferred to a suitable membrane (*e.g.*, Hybond-N®, Amersham) and hybridized to a suitable probe under stringent conditions. The probe should comprise a nucleic acid sequence of a fragment embedded within the desired sequence.

[0082] If the DNA sequence is synthesized chemically, a single stranded oligonucleotide will result. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. While it is possible to chemically synthesize an entire single chain Fv region, it is preferable to synthesize a number of shorter sequences (about 100 to 150 bases) that are later ligated together.

[0083] Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence.

[0084] Once the V_L and V_H DNA sequences are obtained, the sequences may be ligated together so that a single sequence encodes the Fv domain in the form of a single chain antibody. The V_L and V_H sequences may be joined either directly or through a DNA sequence encoding a peptide linker, using techniques well known to those of skill in the art.

[0085] Suitable linkers are well known to those of skill in the art. Linkers may be chosen to facilitate formation of bivalent or multivalent, *e.g.*, triabodies or tetrabodies, forms of the LL2 antibodies. Suitable linkers are at least 1, 2, or 5 amino acids in length, and may be 10, 15, 20 or more amino acids in length. Alternatively, other linkers, such as hinge regions may be used to join the V_H and V_L domains.

[0086] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic

acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Protein sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0087] Nucleic acids encoding single chain anti-CD22 antibodies or variable domains are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, *e.g.*, plasmids, or shuttle vectors. Isolated nucleic acids encoding therapeutic proteins comprise a nucleic acid sequence encoding a therapeutic protein and subsequences, interspecies homologues, alleles and polymorphic variants thereof.

[0088] To obtain high level expression of a cloned gene, such as those cDNAs encoding a suitable anti-CD22 antibody, one typically subclones the gene encoding the anti-CD22 antibody into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable promoters are well known in the art and described, *e.g.*, in Sambrook *et al., supra* and Ausubel *et al., supra*. Eukaryotic expression systems for mammalian cells are well known in the art and are also commercially available. Kits for such expression systems are commercially available.

[0089] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0090] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0091] The nucleic acid comprises a promoter to facilitate expression of the nucleic acid within a cell. Suitable promoters include strong, eukaryotic promoter such as, for example

promoters from cytomegalovirus (CMV), mouse mammary tumor virus (MMTV), Rous sarcoma virus (RSV), and adenovirus. More specifically, suitable promoters include the promoter from the immediate early gene of human CMV (Boshart *et al.*, (1985) *Cell* 41:521) and the promoter from the long terminal repeat (LTR) of RSV (Gorman *et al.*, (1982) *Proc. Natl. Acad. Sci. USA* 79:6777).

[0092] For eukaryotic expression, the construct may comprise at a minimum a eukaryotic promoter operably linked to a nucleic acid operably linked to a polyadenylation sequence. The polyadenylation signal sequence may be selected from any of a variety of polyadenylation signal sequences known in the art, such as, for example, the SV40 early polyadenylation signal sequence. The construct may also include one or more introns, which can increase levels of expression of the nucleic acid of interest, particularly where the nucleic acid of interest is a cDNA (*e.g.*, contains no introns of the naturally-occurring sequence). Any of a variety of introns known in the art may be used.

[0093] Other components of the construct may include, for example, a marker (*e.g.*, an antibiotic resistance gene (such as an ampicillin resistance gene)) to aid in selection of cells containing and/or expressing the construct, an origin of replication for stable replication of the construct in a bacterial cell (preferably, a high copy number origin of replication), a nuclear localization signal, or other elements which facilitate production of the nucleic acid construct, the protein encoded thereby, or both.

[0094] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence may typically be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Such signal peptides would include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[0095] In addition to a promoter sequence, the expression cassette may also contain a transcription termination region downstream of the structural gene to provide for efficient

termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[0096] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase.

[0097] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells.

[0098] Standard transfection methods are used to produce bacterial, mammalian, yeast, insect, or plant cell lines that express large quantities of single chain LL2 antibody or variable region domains, which are then purified using standard techniques (*see, e.g.*, Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

[0099] Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, biolistics, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g.*, Sambrook *et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the anti-CD22 antibody or a variable domain thereof.

[0100] After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of the anti-CD22 antibody or domain. The expressed protein is recovered from the culture using standard techniques.

[0101] The anti-CD22 antibody or variable domain region may be purified to substantial purity by standard techniques known to those of skill in the art, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra;* and Sambrook *et al., supra*).

[0102] The thermal stability and resistance to proteolysis of the purified anti-CD22 antibody or variable domain region may be determined using serum stability assays as described in Example 2 below. For example, the anti-CD22 antibodies are incubated in serum at an appropriate concentration for varying time periods at 37°C. At various time-points, aliquots are collected and tested for binding activity on $CD22^+$ cells.

IV. Immunoconjugates

[0103] One embodiment of the present invention provides a therapeutic molecule, *i.e.*, an immunoconjugate, comprising a stable anti-CD22 antibody of the invention linked to an effector molecule. The anti-CD22 antibody and the effector molecule may be directly adjoined or may be joined through a linker. The effector may be linked to either end of the antibody. Preferably, the effector molecule is a detectable label or a therapeutic molecule such as, for example, a toxin, a small molecule, a cytokine or a chemokine, an enzyme, or a radiolabel. Exemplary toxins are, for example, *Pseudomonas* exotoxin or diphtheria toxin. Suitable toxins are described in *e.g.*, Chaudhary, *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4538, Chaudhary, *et al.* (1989) *Nature* 339:394, Batra, *et al.* (1991) *Mol. Cell. Biol.* 11:2200. Brinkmann, *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8616, Siegall, (1995) *Semin. Cancer Biol.* 6:289. Exemplary small molecules, are, for example, chemotherapeutic compounds such as taxol, doxorubicin, etoposide, and bleiomycin. Exemplary cytokines are, for example, IL-1, IL-2, IL-4, IL-5, IL-6, and IL-12. Suitable cytokines and chemokines are described in, *e.g.*, Rosenblum *et al.* (2000) *Int. J. Cancer* 88:267 and Xu *et al.* (2000) *Cancer Res.* 60:4475 and Biragyn *et al.* (1999) *Nat. Biotechnol.* 17:253. Exemplary enzymes are, for example, RNAses, DNAses, proteases, kinases, and caspases. Suitable proteases are described in, *e.g.*, Bosslet *et al.* (1992) *Br. J. Cancer* 65:234, Goshorn *et al.* (1993) *Cancer Res.* 53:2123, Rodrigues *et al.* (1995) *Cancer Res.* 55:63, Michael *et al.* (1996) *Immunotechnology* 2:47, Haisma *et al.* (1998) *Blood* 92:184. Exemplary RNAses are, for example, RapLR1, and angiogenin. Suitable RNases A family members are described in

.*e.g.*, U.S. Patent No. 5,559,212; WO 99/50398); Newton *et al.* (1994) *J. Biol. Chem.* 269:26739, Newton, *et al.* (1996) *Biochemistry* 35:545, and Zewe, *et al.* (1997) *Immunotechnology* 3:127-136. Exemplary radioisotopes are, for example, ^{32}P and ^{125}I. Suitable radionuclides are also described in, *e.g.*, Colcher *et al.* (1999) *Ann. NY Acad. Sci.* 880:263. Additional exemplary effector moieties are, for example, Fc fragments from homologous or heterologous antibodies.

[0104] When the therapeutic molecule is a protein or polypeptide, it will be appreciated by those of skill in the art that the sequence of the therapeutic molecule may be altered in a manner that does not substantially affect the functional advantages of the therapeutic molecule. For example, glycine and alanine are typically considered to be interchangeable as are aspartic acid and glutamic acid and asparagine and glutamine. One of skill in the art will recognize that many different variations of therapeutic molecule sequences will encode therapeutic molecule with roughly the activity as the native therapeutic molecule. For example, many different variations of RNAse sequences will encode RNAses with the same measurable ribonucleolytic activity as the native RNAse. For example, recombinant *Rana pipiens* RNase A proteins, variants of the proteins, and techniques for synthesizing the proteins are described in WO 99/50398.

[0105] The effector molecule and the anti-CD22 antibody may be conjugated by chemical or by recombinant means (see, Rybak, *et al.*, (1995) *Tumor Targeting* 1:141). Chemical modifications include, for example, derivitization for the purpose of linking the effector molecule and the anti-CD22 antibody to each other, either directly or through a linking compound, by methods that are well known in the art of protein chemistry. In the presently preferred chemical conjugation embodiment, the means of linking the effector molecule and the anti-CD22 antibody comprises a heterobifunctional coupling reagent which ultimately contributes to formation of an intermolecular disulfide bond between the effector molecule and the s antibody. Other types of coupling reagents that are useful in this capacity for the present invention are described, for example, in U.S. Patent 4,545,985. Alternatively, an intermolecular disulfide may conveniently be formed between cysteines in the effector molecule and the anti-CD22 antibody which occur naturally or are inserted by genetic engineering. The means of linking the effector molecule and the antibody may also employ thioether linkages between heterobifunctional crosslinking reagents or specific low pH cleavable crosslinkers or specific protease cleavable linkers or other cleavable or noncleavable chemical linkages. The means of linking the effector molecule and the anti-

CD22 antibody may also comprise a peptidyl bond formed between the effector molecule and the antibody which are separately synthesized by standard peptide synthesis chemistry or recombinant means.

[0106] Exemplary chemical modifications of the effector molecule and the anti-CD22 antibody of the present invention also include derivitization with polyethylene glycol (PEG) to extend time of residence in the circulatory system and reduce immunogenicity, according to well known methods (See for example, Lisi, *et al., Applied Biochem.* 4:19 (1982); Beauchamp, *et al., Anal. Biochem.* **131**:25 (1982); and Goodson, *et al., Bio/Technology* **8**:343 (1990)).

[0107] When the effector molecule is a protein, exemplary recombinant modifications of the proteins of the immunoconjugates include combination of the relevant functional domains of each into a single chain multi-functional biosynthetic protein expressed from a single gene derived by recombinant DNA techniques. (*see, e.g.*, WO 88/09344). Furthermore, recombinant DNA techniques can be used to link the recombinant effector molecule and the anti-CD22 antibody. Accordingly, the immunoconjugate can comprise a fused protein, for example, beginning at one end with the effector molecule and ending with a single chain anti-CD22 antibody.

[0108] Methods of producing recombinant fusion proteins are well known to those of skill in the art. For example, Chaudhary, *et al.* (1989) *Nature* 339:394; Batra, *et al.* (1990) *J. Biol. Chem.* 265:15198; Batra, *et al.* (1989) *Proc. Natl Acad. Sci. USA* 86:8545; Chaudhary, *et al,* (1990) *Proc. Natl Acad. Sci. USA* 87:1066, describe the preparation of various single chain antibody-toxin fusion proteins.

[0109] In some embodiments, producing immunoconjugate fusion proteins involves separately preparing the anti-CD22 Fv light and heavy chains and DNA encoding the protein to be used. The two sequences are combined in a plasmid or other vector to form a construct encoding the particular desired fusion protein. A simpler approach involves inserting the DNA encoding the particular Fv region into a construct already encoding the desired protein. Thus, for example, DNA encoding single chain anti-CD22 antibody/RapLR1 protein immunoconjugates is most easily prepared by inserting the DNA encoding the antibody V_H and V_L chains (Fv region) into constructs already containing DNA encoding the desired RapLR1 protein or vice versa. The DNA sequence encoding the Fv region is inserted into the construct using techniques well known to those of skill in the art.

[0110] Mammalian cells have been used to express and secrete hybrid molecules such as antibody-cytokines (*see, e.g.*, Hoogenboom, *et al.* (1991) *Biochem. Biophys. Acta* 1096:345; Hoogenboom, *et al.*(1991) *Mol. Immunol.* 28:1027) and antibody-enzyme (Casadei, *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:2047; Williams, *et al.* (1986) *Gene* 43:319). In part, immunogenicity of foreign proteins is due to incorrect glycosylation patterns present on recombinant proteins. Therefore, eukaryotic cell lines are preferred over prokaryotic cells as the expressed proteins are glycosylated. Human derived cell lines are particularly preferred in that these cells incorporate a sialic acid as the terminal glycoside. Cell lines such as the hamster CHO and BHK, as well as the HEK-293 human fibroblast line have been used to express recombinant human proteins.

[0111] Other exemplary recombinant modifications of the protein moieties of the immunoconjugates of this invention include deletions of functionally unnecessary domains to reduce the size of the protein or to modify other parameters which facilitate production or utility, such as sequence changes to affect the solubility (*e.g.*, cysteine to serine) or glycosylation sites. One skilled in the art would appreciate that multiple well known chemical and genetic modifications of proteins may be advantageously applied to any protein which, like the immunoconjugate of the present invention, may be used for parenteral administration.

[0112] The immunoconjugate of the present invention may also be utilized for selective killing of malignant cells, *e.g.*, malignant cells that overexpress CD22 on their cell surface. The LL2 antibody will target the immunoconjugate to malignant cells overexpressing CD22 on their surface, thus delivering the therapeutic molecule to the cell and killing the cell.

D. Pharmaceutical Compositions

[0113] According to one embodiment of the present invention, a stable anti-CD22 antibody of the invention, or an immunoconjugate comprising the antibody can be used to inhibit the growth of malignant B cells. In one embodiment the immunoconjugates of the present invention can be used as part of a therapeutic regimen to treat multiple diseases. One preferred embodiment of the present invention is the use of the immunoconjugates in the treatment of malignant B cells expressing CD22. Exemplary malignant diseases of B cells include acute lymphocytic leukemia (ALL), chronic B-lymphocytic leukemia (B-CLL),

chronic myelogenous leukemia, Burkitt's, AIDS-associated and Follicular lymphomas, and hairy cell leukemias.

[0114] The present invention also relates to a pharmaceutical composition comprising immunoconjugates of the present invention in a pharmaceutically acceptable carrier. In therapeutic applications, compositions are administered to a patient suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a therapeutically effective dose. Amounts effective for this use will depend on the severity of the disease and the general state of the patient's health.

[0115] Advantageously, the pharmaceutical composition is suitable for parenteral administration. The immunoconjugates of the present invention may be administered by various means appropriate for different purposes, for example, for treating tumors in various parts of the body, according to methods known in the art for other immunoconjugates. (*See, e.g.*, Rybak, *et al.*, Human Cancer Immunology, in IMMUNOLOGY AND ALLERGY CLINICS OF AMERICA, W. B. Saunders, 1990, and references cited therein). Accordingly, the present invention also relates to pharmaceutical compositions comprising an immunoconjugates of the present invention and a pharmaceutically acceptable carrier, particularly such compositions which are suitable for the above means of administration.

[0116] Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient.

[0117] Preferably, the compositions for administration comprise a solution of the immunoconjugate comprising the anti-CD22 antibody and the therapeutic molecule, *e.g.*, RNAse dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, sterilization techniques known in the art. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of immunoconjugate in these formulations can vary

widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

[0118] Thus, a typical pharmaceutical composition for intravenous administration would be about 0.01 to 100 mg per patient per day. Dosages from 0.1 up to about 1000 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a tumor or an organ within which a tumor resides. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as REMINGTON'S PHARMACEUTICAL SCIENCE, 17TH ED., Mack Publishing Co., Easton, PA, (1985).

[0119] Typically, the pharmaceutical compositions containing the immunoconjugates are administered in a therapeutically effective dose over either a single day or several days by daily intravenous infusion.

[0120] The immunoconjugates of the present invention may be administered systemically by injection, most preferably intravenously, but also intramuscularly, subcutaneously, intrathecally, intraperitoneally, into vascular spaces, or into joints, *e.g.*, intraarticular injection. The dose will be dependent upon the properties of the immunoconjugate employed, *e.g.*, its activity and biological half-life, the concentration of the immunoconjugate in the formulation, the site and rate of dosage, the clinical tolerance of the patient involved, the extent of cancer afflicting the patient and the like as is well within the skill of the physician.

[0121] The immunoconjugate of the present invention may be administered in solution. The pH of the solution should be in the range of pH 5 to 9.5, preferably pH 6.5 to 7.5. The immunoconjugates or derivatives thereof should be in a solution having a suitable pharmaceutically acceptable buffer such as phosphate, tris (hydroxymethyl) aminomethane-HCl or citrate and the like. Buffer concentrations should be in the range of 1 to 100 mM. The solution of the immunoglobulin may also contain a salt, such as sodium chloride or potassium chloride in a concentration of 50 to 150 mM. An effective amount of a stabilizing agent such as albumin, a globulin, a detergent, a gelatin, a protamine or a salt of protamine may also be included and may be added to a solution containing the immunoconjugate or to the composition from which the solution is prepared. Systemic administration of the immunoconjugate is typically made every two to three days or once a week if a humanized

form of the antibody is used. Alternatively, daily administration is useful. Usually administration is by either intramuscular injection or intravascular infusion.

[0122] Administration may also be intranasal or by other nonparenteral routes. The immunoconjugate may also be administered via microspheres, liposomes or other microparticulate delivery systems placed in certain tissues including blood.

[0123] The immunoconjugate may also be administered by aerosol to achieve localized delivery to the lungs. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing or derivatives thereof. A nonaqueous (*e.g.*, fluorocarbon propellent) suspension could be used. Sonic nebulizers preferably are used in preparing aerosols. Sonic nebulizers minimize exposing the antibody or derivatives thereof to shear, which can result in degradation of the immunoconjugate.

[0124] Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the immunoconjugate together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers will vary depending upon the requirements for the particular immunoconjugate, but typically include nonionic surfactants (TWEEN-20 OR -80®, PLURONIC-F128 OR -67®, or polyethylene glycol), innocuous proteins like serum albumin, or sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. The formulations will be sterile. Aerosols generally will be prepared from isotonic solutions.

[0125] One embodiment of the present invention provides a method of selectively killing cells using an immunoconjugate. Binding of the anti-CD22 antibody to the CD22 surface marker on a malignant B cell causes the therapeutic reagent to selectively kill the cell. The pharmaceutical composition described above can conveniently be used to deliver the immunoconjugate of the present invention.

V. Detecting Cells Expressing CD22

[0126] According to another embodiment of the present invention, the stable anti-CD22 antibodies and/or immunoconjugates comprising the antibodies can be used to detect cells expressing the cell surface molecule CD22. Such uses may be diagnostic, *e.g.*, detecting $CD22^+$ malignant cells, or therapeutic, *e.g.*, inhibiting proliferation of malignant $CD22^+$ cells. *In vivo* methods of detecting cells using antibodies are well known in the art and are described, for example, in U.S. Patent Nos. 6,375,925, 6,348,581, 6,333,405, and 6,281,335.

In vitro immunoassays using antibodies to detect cell surface molecules are well known in the art and are described, for example, in *Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7th ed. 1991).

[0127] Preferably the anti-CD22 antibodies or immunoconjugates are linked to a detectable label. The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS™), fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (e.g., polystyrene, polypropylene, latex, etc.).

[0128] The label may be coupled directly or indirectly to the anti-CD22 antibody component of the immunoconjugate according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the antibody, stability requirements, available instrumentation, and disposal provisions.

[0129] Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the anti-CD22 antibody. The ligand then binds to another molecules (*e.g.*, streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*,

luminol. For a review of various labeling or signal producing systems that may be used, see U.S. Patent No. 4,391,904.

[0130] Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Colorimetric or chemiluminescent labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

[0131] Throughout the *in vitro* assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, optionally from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, antigen, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

EXAMPLES

[0132] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Cloning of Single Chain LL2 and Generation of LL2 Variants

[0133] Based on the published sequence of the variable domains for the monoclonal antibody LL2 (Leung *et al.*, 1994), the genes for the variable heavy chain (V_H) and the variable light chain (V_L) were amplified by overlap extension PCR techniques using the following primers which are designed to amplify overlapping regions of the V_H and V_L domains:

LL2-Backward-Sfi I

5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTCCAGCTGCAGGAG-3'

LL2-Backward #1

5'CAGGTCCAGCTGCAGGAGTCAGGGGCTGAACTGTCAAAACCTGGGGCCTCAGTGAAGATGTCCTGCA
AGGCTTCTGGCTACACCTTTACT-3'

LL2-Backward #2
5'GCTTCTGGCTACACCTTTACTAGCTACTGGCTGCACTGGATAAAACAGAGGCCTGGACAGGGTCTGG
AATGGATTGGA-3'

LL2-Backward #3
5'TACATTAATCCTAGGAATGATTATACTGAGTACAATCAGAACTTCAAGGACAAGGCCACATTGACTG
CA GACAAATCCTCCAGCACAGCCT-3'

LL2-Backward #4
5'AAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGACATCTGAGGACTCTGCAGTCTATTACT
GCGCGCGA-3'

LL2-Backward #5
5'GCAGTCTATTACTGCGCGCGAAGGGATATTACTACGTTCTACTGGGGCCAAGGCACCACTCTCACAG
TCTCCTCG-3'

LL2-Backward #7
5'-GGGTCTGGAATGGATTGGATACATTAATCCTAGGAAT-3'

LL2-Forward #8
5'-ATTCCTAGGATTAATGTATCCAATCCATTCCAGACCC-3'

LL2-Forward-NotI
5'-TTCTCGACTTGCGGCCGCCGAGGAGACTGTGAGAGTGG-3'

[0134] To obtain the wild-type LL2 scFV (referred to herein as scFv MLZ-wt), a sequence encoding for the (Gly4Ser)3 linker was introduced between the V_H and V_L domain. The scFv gene was fused at its C-terminal end with a c-myc and a hexahistidine epitope tag by cloning into the bacterial expression plasmid pHOG21 (Kipriyanov, *et al.*, *J Immunol Methods* 196:51-62, 1996). using vector compatible restriction sites NcoI/BamHI. Variants of the wildtype scFV, which are further described below, were generated by site-directed mutagenesis and overlap extension PCR techniques (*see, e.g.*, Ho *et al.*, *Gene* 77:51-59, 1989). Vectors were transformed in the *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA) for sequencing. Sequences of MLZ and the generated variants were verified by automated DNA sequencing.

Periplasmic expression and purification of scFv fragments

[0135] Starting from a single colony of the E. coli strain TG1 (Stratagene, La Jolla, CA) transformed with the respective scFv expression plasmid a one liter bacterial culture was grown at 37°C in 2xYT medium containing 100 µg/ml ampicillin and 100 mM glucose to an OD600 of 0.9. Bacteria were pelleted by centrifugation at 1500 x g for 20 min at 20°C and resuspended in the same volume of fresh 2xYT medium containing 100 µg/ml ampicillin, 0.4 M sucrose and 1mM IPTG. The culture was grown at 19°C overnight. Bacteria were harvested at 7,000 x g for 30 min at 4°C and the pellets resuspended in 5% of the initial volume in ice cold periplasmic extraction buffer (50mM Tris, 1mM EDTA, 20% Sucrose; pH 8.0) and incubated for 1 h on ice. Supernatant containing the soluble scFv was cleared by centrifugation at 30,000 x g for 60 min at 4°C and thoroughly dialyzed against SP-20 buffer (300 mM NaCl, 50 mM NaH2PO4, 20 mM imidazole; pH8.0). The dialyzed crude periplasmic extract was purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA (Ni-nitrilotriacetic acid (Qiagen, Valencia, CA) according to the manufacturers protocol.

[0136] Monomeric scFv fragments were isolated by size-exclusion chromatography using a calibrated Superdex 75 HR 10/30 column (Amersham Pharmacia, Piscataway, NJ). Purified antibodies were analyzed on 4-20% SDS-PAGE under reducing conditions and stained with Simply Blue™ Safe Stain (Invitrogen, Carlsbad, CA) or by Western blot analysis using anti-c-myc mAb 9E10 (Roche, Indianapolis, IN) as first and anti-mouse IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) as secondary antibody, followed by BCIP/NBT purple liquid substrate (Sigma) detection. Concentrations of purified scFvs were determined by measuring the absorbance at A280nm in a spectrophotometer assuming that 1 A280nm corresponds to 0.5 mg/ml protein as calculated by the software program Gene Inspector (Textco, West Lebanon, New Hampshire). For N-terminal sequence analysis by Edman degradation the protein bands obtained by SDS-PAGE were transferred onto a PVDF membrane (Biorad, Hercules,CA), stained with Coomassie brilliant blue, and sequenced using an Applied Biosystems Model 494 CLC Precise sequenator. The yield of the wildtype monomeric MLZ-wt scFv was only 10 µg/L.

Identification of residues for generation of variants

[0137] To identify important residues that contribute to the very poor stability of the wild-type single chain LL2, its sequence was analyzed. The antibody variable domain subgroups

were shown to be subgroup IIb for V_H and subgroup I for V_L, which is in contrast to the previous classification of V_L as subgroup V (Leung *et al.*, *supra*). Based on homology to members of the particular subgroup, several amino acid positions residues were identified as potentially affecting the thermal stability of the wildtype LL2 scFV. These six residues of scFv MLZ-wt at positions V_H5, V_H12, V_H62, V_L4, V_L18, and V_L77, did not match invariant consensus residues of the identified subgroups. Furthermore, one uncommon interface residue at postion VLH89 was detected (Table 1).

Table 1. Uncommon residues in MLZ-wt

	V_H			V_L			
	6 FR1	12 FR1	62 CDR2	4 FR1	18 FR1	77 FR3	89 CDR3
MLZ-wt	E	S	N	L	N	R	H
consensus	Q	V	K	M	K	S	Q/L/F

[1]Residue numbering according to Kabat *et al.*, *supra*; FR, framework region; CDR, complementarity-determining region.

[0138] The uncommon residues V_H6, V_H12, V_H62, V_L4, V_L18, and V_L77 were further assigned to their structural role within the common core of immunoglobulin variable domains (Chothia *et al*, *J Mol Biol.* 278:457-479, 1998). Since V_L18 and V_L77 are not part of the set of sites which form the interior of the core where residues with only low restrictive conservation of chemical classes are found, these two sites were not considered candidates for mutagenesis to respective consensus amino acids.

[0139] Residue V_L4 belongs to a set of 15 highly conserved sites forming the central hydrophobic region of the core (Chothia, *et al.*, *supra*) and non-conservative substitutions at these sites can have notable effects on the domain stability. Because this is a potentially important site and because the leucine at position four of MLZ-wt could have been introduced by the PCR primer used for amplification of the variable heavy chain (Leung *et al.*, *supra*), this site was included for homology modeling, further described below, in order to study possible effects on mutagenesis of V_LL4→ M.

[0140] Residue V_H6 is located at one of the two buried polar regions adjacent to the central hydrophobic region (Chothia *et al*, *supra*). The strongly conserved residues of this region are important for the conformation of the bulged G strand, which is crucial for the V_H/V_L interface formation (Chothia *et al.*, *J Mol Biol.* 1985;186:651-663, 1985). and for the framework I conformation (Honegger & Pluckthun, *J Mol Biol.* 309:687-699, 2001). Thus,

the glutamic acid at V_H6 of MLZ-wt was considered to have a high potential to destabilize the wild-type scFv.

[0141] Residue V_H12 is a member of sites at the peripheral buried structure of the central core where a less pronounced conservation of residues can be found (Chothia *et al., supra*). VH12 is in part accessible to the surface with its hydrophobic side chains packed between the β-sheets. Serine in the wild type sequence is extremely rare at this location and not present in any germline sequence (Chothia *et al, supra*). Potentially stabilizing effects of consensus residue mutagenesis $V_HS12 \rightarrow V$ were therefore analyzed using a homology model.

[0142] Residue V_H62 is located within the CDR2 region 23, but not directly involved in the structural antigen binding loop H2 33. V_H62 is located adjacent to the deep structure of the common core and buried by the loop D(Chothia *et al, supra*). Possible effects on the stability of the scFv antibody by substitution of V_H62 with the consensus amino acid were also considered by computer homology modeling.

[0143] The effects of the scFv stability by mutagenesis of V_H6, V_H12, V_H62, V_L4, and V_L89 to respective consensus residues were analyzed using a homology model to scFv 1F9 (Ay *et al., J Mol Biol.* 301:239-246, 2000), which aligned with the highest score of -107.6 and a sequence identity of 67% over 239 residues.

[0144] The homology model showed that V_LH89 is not exposed to the V_H/V_L interface and does not form a direct interaction with its corresponding interface residue. Thus, this residue was not considered to negatively influence the stability of the wild-type scFv and was not substituted with the consensus residue in the analysis described below. The homology model showed V4 as pointed into the V_L core. Mutagenesis of $V_LL4 \rightarrow M$ appeared to be conservative and was predicted to have no additional stabilizing effect on the molecule. Exchange at V_H6 from glutamate to the consensus residue glutamine was shown to result in an additional hydrogen bond of the V_H6Q-NE2 to the carbonyl oxygen of V_HY90, which clearly predicted a gain in stability. Modeling of V_H12, which is located adjacent to the linker region, suggested an exchange of the hydrophilic serine with the hydrophobic valine side chain to result in additional anchoring to the linker region. Additionally, exchanging asparagine for lysine of V_H62 was predicted to create a hydrogen bonding network mediated by a water molecule between the side chains of V_HK62, V_HR40, and V_HE85 of the variable heavy chain. Variants were therefore generated based on this analysis.

Variant LL2 scFv antibodies

[0145] Variant single chain LL2 antibody variants were generated by site directed mutagenesis to exchange these residues. Specifically, single chain LL2 variants with substitutions at one or more of: positions 6, 12, and 62 of the V_H chain and positions 4 and 25 of the V_L chain. Of these, the three residues in the V_H region were identified as particularly important residues for substitution.

[0146] Tables 2a and 2b below shows substitutions introduced to generate particular LL2 variants. Table 2a shows substitutions that were introduced into the V_H region only at the designated positions. Table 2b shows substitutions in V_H and V_L at the designated positions.

Table 2a. The ♦ symbol indicates that the variant possesses the designated substitution.

Variant	V_H6 E→Q	V_H12 S→V	V_H62 N→K	V_L4 L→M
MLZ-wt				
MJ-1				♦
MJ-2			♦	
MJ-3		♦		
MJ-4	♦			
MJ-5	♦		♦	
MJ-6	♦	♦		
MJ-7	♦	♦	♦	
MJ-8	♦	♦	♦	♦

Table 2b. The designation "MLV5 (wt)" is equivalent to MLZ-wt; the designation "MLV6.1" is equivalent to MJ-7; and the designation "MLV8.1" is equivalent to MJ-6.

	V_H Pos 6	V_H Pos 12	V_H Pos 62	V_L Pos 4	V_L Pos 25
MLV5 (wt)	E	S	N	L	S
MLV 5.1	Q	V	K	L	A
MLV 6.1	Q	V	K	L	S
MLV 8.1	Q	V	N	L	S
MLX 2.1	Q	V	K	M	A
MLV 11.2*	Q	V	K	M	S
MLV 3.3	Q	S	K	M	A
MLV 2.10	Q	S	N	M	A
MLV 1.1	E	S	K	M	A
MLV 7.1	E	S	N	L	S
MLV 10.2*	E	S	N	L	S
MLV 4.1	E	S	N	M	A

*MLV 11.2 and MLV 10.2 also have a substitution of V for Q at position 3 of the V_L domain.

[0147] Variants were generated using site directed mutagenesis using the following primers

VH-1.1
5'-TATAGCCATGGCGCAGGTCCAGCTGCAGCAGTCAGGGGCTGAACTGGCAAAACCTGGGGCC-3'

VH-1.2
5'-TATAGCCATGGCGCAGGTCCAGCTGCAGCAGTCAGGGGC-3'

VH-12V
5'-TATAGCCATGGCGCAGGTCCAGCTGCAGCAGTCAGGGGCTGAACTGGTGAAACCTGGG-3'

VH-1.3.1
5'TATAGATATCTGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCCGAGGAGACTGT
GAGAGTGGTGCCTTGGCCCC-3'

VH- 62Kforward
5'-GTACAATCAGAAATTCAAGGACAAGG-3'

VH 62Kbackward
5'-CCTTGTCCTTGAATTTCTGATTGTAC-3'

VL-2.1.1
5'-TATAGATATCCAGCTGACCCAGTCTCCATCATCTCTGGC-3'

VL-2.2.1
5'GTCTCCATCATCTCTGGCTGTGTCTGCAGGAGAAAACGTCACTATGAGCTGTAAGTCCAGTCAAAGT
GTTTTATACAGTGCAAATCACAA-3'

VL-2.3
5'CTAGTGGATGCCCAGTAGATCAGCAGTTTAGGAGACTGCCCTGGTTTCTGCTGGTACCAGGCCAAGT
AGTTCTTGTGATTTGCACTGTAT-3'

VL-2.4
5'-TCAGGGACACCAGATTCCCTAGTGGATGCCCAGTAG-3'

VL-2.5
5'-CTACTGGGCATCCACTAGGGAATCTGGTGTCCCTGA-3'

VL-2.6
5'GGAATCTGGTGTCCCTGATCGCTTCACAGGCAGCGGATCTGGGACAGATTTTACTCTTACCATCAGC
AGAGTACAAGTTGAAGACCTGGC-3'

VL-2.7
5'ACGTTTGATCTCCAGCTTGGTCCCTCCACCGAACGTCCACGAGGAGAGGTATTGGTGACAATAATAA
ATTGCCAGGTCTTCAACTTGT-3'

VL-2.9
5'-TATAGGATCCACGTTTGATCTCCAGCTTGGTCCCTCC-3'

VL-2.1.2
5'-TATAGATATCCAGATGACCCAGTCTCCATCATCTCTGGC-3'

VL-2.2.2
5'GTCTCCATCATCTCTGGCTGTGTCTGCAGGAGAAAACGTCACTATGAGCTGTAAGGCCAGTCAAAGT
GTTTTATACAGTGCAAATCACAA-3'.

Example 2. Characterization of variants

[0148] The influence of substitutions introduced at the identified positions, alone and in various combinations, on solubility, binding activity and stability of the variant LL2 scFv was then determined. All scFv variants were isolated as soluble proteins from the periplasm of *E. coli* and purified by IMAC as described above.

Cell binding analysis and competition assay

[0149] CD22 expressing human B cell lines Raji, Ramos, Daudi, and CA46 (American Type Culture Collection, Manassas, VA) were used to determine the specific binding of the constructs and CD22- T cell lines Jurkat and HUT102 (American Type Culture Collection) served as negative controls. For binding analysis, aliquots of 5x 10^5 cells were incubated for 45 min at 4°C in round bottom 96-well microtiter plates with 100 µl of a sample containing the scFv fragment or control antibodies in FACS buffer (PBS, 0.1 % NaN_3, 2% FBS). Cells were pelleted at 200 x *g* for 5 min at 4°C and washed twice with 200 µl FACS buffer. For detection of bound antibodies cells were first incubated for 30 min at 4°C with saturating concentrations of the anti-c-myc mAb 9E10 (10 µg/ml; Roche) followed by two washes and incubation with saturating amounts of FITC-labeled anti-mouse IgG (13 µg/ml; Jackson Immuno Research, West Grove, PA) for 30 min at 4°C. To exclude dead cells from the analysis, cells were washed as above and resuspended in FACS buffer containing 10 µg/ml propidium iodide (Sigma). Background fluorescence was determined by using cells incubated with 9E10 antibody and fluorescein-labeled anti-mouse antibody under the same conditions. Stained cells were analyzed on a FACScan Flow Cytometer (BD Bioscience, San Jose, CA), and the median fluorescence intensity (MFI) was calculated using the CellQuest™

software (BD Bioscience). For competition experiments, Raji cells were preincubated with increasing concentrations of scFv in FACS buffer or FACS buffer alone for one hour at 4°C. After adding the mAb LL2 at a 100-fold molar excess of the scFv starting concentration, cells were incubated for an additional hour at 4°C. After two washes with FACS buffer, bound LL2 was detected using FITC-labeled anti-mouse IgG and samples were analyzed as described above. The inhibition of LL2 binding to Raji cells by competitive scFv was determined as percentage of maximal MFI of LL2 in the absence of competing scFv antibodies.

Determinantion of affinity constants (Kd)

[0150] Binding affinity constants were determined as previously described [31] with the following modifications: Varying concentrations of antibodies (0.5 nM - 2 μM) were incubated in triplicates with 5x 10^5 Raji cells at room temperature in FACS buffer for two hours. Bound antibodies were detected under the same conditions as before. After two final washing steps cells were fixed in PBS containing 2% paraformaldehyde for 15 minutes at room temperature and analyzed by flow cytometry. The MFI was determined as described above and background fluorescence was subtracted. Equilbrium constants were determined by using the Marquardt and Levenberg method for non-linear regression with the GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diago, CA).

Biophysical stability analysis

[0151] Aliquots of scFv variants were incubated at concentrations of 10 μg/ml in 90% human serum for varying periods of time at 37°C. Samples were frozen at –20°C at different time points until the end of the experiment. Samples were subsequently analyzed for binding activity to $CD22^+$ Raji cells by flow cytometry. The half-life was determined as the time point with 50% of the initial binding activity.

Fluorescence-labeling and internalization experiments of mAb LL2 and scFv MJ-7

[0152] Internalization studies were conducted using 100 μg of mAb LL2 and 50 μg of the scFv-fragment, which were labeled with Alexa Fluor 488 using a monoclonal antibody labeling kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. To purify labeled scFv from free Alexa Flour 488 dye the reaction mixture was thoroughly dialyzed against PBS. The labeled mAb was separated from unbound dye by gel filtration. Specific binding of labeled protein was assayed on $CD22^+$ Raji and $CD22^-$ Jurkat cells by flow cytometry analysis. For internalization studies 5x10^5 Raji cells were incubated with 50

μg/ml of Alexa 488 labeled antibody for 30 min on ice, followed by incubation at 37°C for various times. At time points 0, 30, and 60 min cells were washed twice with PBS and analyzed for membrane binding or internalization by confocal microscopy using an Insight Plus laser scanning system (Meridian Instruments, Inc., Okemos, MI) equipped with an Olympus IMT-2 inverted microscope.

Serum Stability of Single Chain LL2 Variants in comparison to wildty type LL2-scFv

[0153] For initial characterization, expressed scFv variants were purified by IMAC. Flow cyotmetry studies demonstrated a specific binding of all purified scFv mutants to $CD22^+$ expressing cells and non-binding to CD22 negative control cells (see, Figure 2A for a representative experiment). Flow cytometric experiments also demonstrated a specific binding of the scFv MLZ-wt to several $CD22^+$ cell lines and non binding to $CD22^-$ control cell lines. However, the freshly purified scFv MLZ revealed a very low biophysical stability with complete loss of its initial binding activity after only 4 h incubation in human serum at 37°C (Figure 2B, comparing wildty-scFV and stable scFV binding).

[0154] The half lives of particular single chain LL2 variants are shown in Figures 3A and 3B and in Table 2. The most stable constructs, MLV5.1 and MLV6.1 (also referred to as MJ-7), showed specific binding to $CD22^+$ cells even after extended periods of incubation. Variant MJ-1 with single mutation VLL4→ M exhibited an even shorter half-life with 0.25 h than the wild-type MLZ (0.6 h). Among the variants with single amino acid substitutions in the heavy chain only variant VHE6→ Q showed a significant higher stability (13-fold) than MLZ-wt. A 21 and 50-fold increase in stability was achieved with the double mutants MJ-5 (VHE6→ Q/N62→ K) and MJ-6 (VHE6→ Q/S12→ V), respectively. Clearly synergistic effects on stability of amino acid exchanges at all three VH positions were shown demonstrated for MJ-7 (VHE6→ Q/S12→ V/N62→ K), which exhibited an 83.3-fold improved half-life when compared with the wild-type MLZ. Indeed, MJ-7 did not reach its half live when incubated in human serum at 37°C for 6 days and retained its immunoreactivity with tumor cells when stored in PBS/50 mM imidazole at 4°C for >10 months.

Table 2

LL2 Variant	Half-Life (hours)
MLV5 (wt)	0.6
MLV 5.1	52
MLV 6.1	50
MLV 8.1	30
MLX 2.1	22.5
MLV 11.2	20
MLV 3.3	13
MLV 2.10	8
MLV 1.1	0.75
MLV 7.1	0.7
MLV 10.2	0.4
MLV 4.1	0.25

[0155] To compare one of the most stable constructs with the wild-type scFv *in vitro*, monomers of MJ-7 were purified by size exclusion chromatography. Yield of purified MJ-7 (200 ug/L culture) was 20-fold higher than the MLZ-wt and no significant degradation was observed (Figure 4A).

[0156] Incubation of monomeric MJ-7 in human serum for up to six days at 37°C showed that the engineered variant retained 80% immunoreactivity with $CD22^+$ tumor cells after an incubation period of six days, whereas the wild-type scFv MLZ rapidly lost its binding activity (Figure 4B).

Example 3: Flow Cytometry Analysis of LL2 Variants

[0157] As noted above, all single chain LL2 variants shown in Table 1a and 1b showed specific binding to $CD22^+$ cells (*see, e.g.*, Figure 3). To test whether the introduced consensus residue mutations affected antigen binding properties, antigen affinity constants and epitope specificity of MJ-7 were determined in comparison with MLZ-wt. The mutant MJ-7 bound CD22 on Raji cells with an even slightly higher affinity (K_D 16.6 ±0.35 nM) when compared with the MLZ wild-type scFv (K_D 24.3 ±1.3 nM) (Figure 5A). In competition experiments a 20-fold molar excess of either scFv MJ-7 or wild-type MLZ, respectively, blocked the binding of the mAb LL2 to tumor cells by 95% (Figure 5B) indicating that the stability engineered MJ-7 retained its full specificity.

[0158] The affinity of other single chain LL2 antibodies is determined using similar methodology.

Binding and internalization

[0159] To assess the therapeutic potential of the engineered scFv to deliver cytotoxic agents into tumor cells, MJ-7 was labeled with the fluorescent dye Alexa-488 and internalization into living Raji cells was monitored by confocal microscopy. Receptor-mediated endocytosis of the engineered MJ-7 fragment was as efficient as for the mAb LL2. Internalization into Raji cells of both the parental antibody LL2 and scFv MJ-7 became visible after only 5 min incubation at 37°C. After 30 min at 37°C both antibodies were internalized by >90%. Internalization was clearly dependent on temperature since Raji cells incubated with the antibodies for 30 min on ice showed a much slower internalization rate.

[0160] These results show that mutagenesis of the identified destabilizing amino acids to corresponding consensus residues markedly stabilized the molecule but did not influence its antigen binding properties. In particular, the engineered scFv MJ-7 (also referred to as MLV6.1) and conservative modifications of this antibody, *e.g,* MLV5.1) exhibited exceptional biophysical stability with a half-life not reached after six days of incubation in human serum at 37°C, fully retained the Epitope specificity of the murine ancestor, and bound to the target antigen with an affinity comparable to the wild-type scFv. Furthermore, the monoclonal antibody LL2 and the engineered scFv fragment became both fully internalized after only 30 min of incubation at 37°C with $CD22^+$ tumor cells.

Additional engineering of scFV

[0161] To further stabilize the scFv antibody, an scFv antibody fragment with engineered intermolecular disulfide bonds was constructed: The fragment specifically binds to target cells.

[0162] A bivalent diabody from the stable scFv mutant MJ-7 was also generated: the diabody has a V_H/V_L orientation and both domains are connected by a 5 amino acid peptide linker (GGGS). The diabody specifically binds to target cells with a binding affinity about 3-fold higher than scFv, as expected. The diabody has a half live when incubated in human serum at 37°C of 12h.

[0163] A zero linker construct in a V_L-V_H orientation also showed specific binding to $CD22^+$ tumor cells. In size exclusion chromatography this fragment elutes as a dimer. The affinity is similar to the scFv (and not, as expected, as good as for the diabody). Surprisingly, stability assays with purified dimers revealed an 8-fold better stability of this construct than

the diabody construct. In contrast to the scFv and the diabody, this fragment is not cleaved by *E. coli* proteases.

[0164] Figure 6 illustrates flow bytometric analysis of the V_L-V_H zero linker construct, a stable single chain LL2 antibody variant MLV5.1, and the monoclonal antibody LL2.

[0165] Fusion proteins were also generated comprising the human ribonuclease Angiogenin and engineered scFv/diabody/0-linker diabody.

[0166] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

[0167] All publications, patents, accession numbers, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

Table of Sequences

SEQ ID NO:1
PRT wild type LL2 VH-VL

QVQLQESGAELSKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQNFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPSSLAVSAGENV
TMSCKSSQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:2
PRT variant MLV 5.1

QVQLQQSGAELVKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQKFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPSSLAVSAGENV
TMSCKASQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:3
PRT variant MLV 6.1

QVQLQQSGAELVKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQKFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPSSLAVSAGENV
TMSCKSSQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:4
PRT variant MLV 8.1

QVQLQQSGAELVKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQNFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPSSLAVSAGENV
TMSCKSSQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:5
PRT variant MLX 2.1

QVQLQQSGAELVKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQKFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLAVSAGENV
TMSCKASQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:6
PRT variant MLV 11.2

QVQLQQSGAELVKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQKFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQSPSSLAVSAGENV
TMSCKSSQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:7
PRT variant MLV 3.3

QVQLQQSGAELSKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQKFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLAVSAGENV
TMSCKASQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:8
PRT variant MLV 2.10

QVQLQQSGAELSKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQNFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLAVSAGENV
TMSCKASQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:9
PRT variant MLV 1.1

QVQLQESGAELSKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQKFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLAVSAGENV
TMSCKASQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:10
PRT variant MLV 7.1

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TMSCKSSQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:11
PRT variant MLV 10.2

QVQLQESGAELSKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQNFKDKATLTADKS
SSTAYMQLSSLTSEDSAVYYCARRDITTFYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQSPSSLAVSAGENV
TMSCKSSQSVLYSANHKNYLAWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISRVQVEDLAIYYC
HQYLSSWTFGGGTKLEIKR

SEQ ID NO:12
PRT variant MLV 4.1

QVQLQESGAELSKPGASVKMSCKASGYTFTSYWLHWIKQRPGQGLEWIGYINPRNDYTEYNQNFKDKATLTADKS
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HQYLSSWTFGGGTKLEIKR

SEQ ID NO:13
DNA wild type LL2 VH-VL

CAGGTCCAGCTGCAGGAGTCAGGGGCTGAACTGTCAAAACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
GGCTACACCTTTACTAGCTACTGGCTGCACTGGATAAAACAGAGGCCTGGACAGGGTCTGGAATGGATTGGATAC
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GGCAGCGGATCTGGGACAGATTTTACTCTTACCATCAGCAGAGTACAAGTTGAAGACCTGGCAATTTATTATTGT
CACCAATACCTCTCCTCGTGGACGTTCGGTGGCGGGACCAAGCTGGAGATCAAACGT

SEQ ID NO:14
DNA variant MLV 5.1

CAGGTCCAGCTGCAGCAGTCAGGGGCTGAACTGGTGAAACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
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AAACCAGGGCAGTCTCCTAAACTGCTGATCTACTGGGCATCCACTAGGGAATCTGGTGTCCCTGATCGCTTCACA
GGCAGCGGATCTGGGACAGATTTTACTCTTACCATCAGCAGAGTACAAGTTGAAGACCTGGCAATTTATTATTGT
CACCAATACCTCTCCTCGTGGACGTTCGGTGGCGGGACCAAGCTGGAGATCAAACGT

SEQ ID NO:15
DNA variant MLV 6.1

CAGGTCCAGCTGCAGCAGTCAGGGGCTGAACTGGTGAAACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
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TCCAGCACAGCCTATATGCAACTGAGCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGCGCGCGAAGGGAT
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AAACCAGGGCAGTCTCCTAAACTGCTGATCTACTGGGCATCCACTAGGGAATCTGGTGTCCCTGATCGCTTCACA
GGCAGCGGATCTGGGACAGATTTTACTCTTACCATCAGCAGAGTACAAGTTGAAGACCTGGCAATTTATTATTGT
CACCAATACCTCTCCTCGTGGACGTTCGGTGGCGGGACCAAGCTGGAGATCAAACGT

SEQ ID NO:16
DNA variant MLV 8.1

CAGGTCCAGCTGCAGCAGTCAGGGGCTGAACTGGTGAAACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
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ATTAATCCTAGGAATGATTATACTGAGTACAATCAGAACTTCAAGGACAAGGCCACATTGACTGCAGACAAATCC
TCCAGCACAGCCTATATGCAACTGAGCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGCGCGCGAAGGGAT
ATTACTACGTTCTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCGGGTGGAGGCGGTTCAGGCGGAGGTGGC
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AAACCAGGGCAGTCTCCTAAACTGCTGATCTACTGGGCATCCACTAGGGAATCTGGTGTCCCTGATCGCTTCACA
GGCAGCGGATCTGGGACAGATTTTACTCTTACCATCAGCAGAGTACAAGTTGAAGACCTGGCAATTTATTATTGT
CACCAATACCTCTCCTCGTGGACGTTCGGTGGCGGGACCAAGCTGGAGATCAAACGT

SEQ ID NO:17
DNA variant MLX 2.1

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SEQ ID NO:25
DNA synthetic

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SEQ ID NO: 26
PRT synthetic

GGGGSGGGGSGGGGS

SEQ ID NO:18
DNA variant MLV 11.2

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SEQ ID NO:19
DNA variant MLV 3.3

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SEQ ID NO:20
DNA variant MLV 2.10

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SEQ ID NO:21
DNA variant MLV 1.1

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SEQ ID NO:22
DNA variant MLV 7.1

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SEQ ID NO:23
DNA variant MLV 10.2

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SEQ ID NO:24
DNA variant MLV 4.1

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WHAT IS CLAIMED IS:

1. A stable anti-CD22 antibody having a V_H domain and a V_L domain, wherein the sequences of the V_H and V_L domains are at least 90% identical to the V_H and V_L sequences set forth in SEQ ID NO:1; and wherein the antibody comprises at least two of the following amino acid substitutions with respect to SEQ ID NO:1: Gln replacing Glu at position 6 of the V_H domain; Val replacing Ser at position 12 of the V_H domain; or Lys replacing Asn at position 62 of the V_H domain.

2. The antibody of claim 1, comprising all three of the substitutions.

3. The antibody of claim 2, further comprising an Ala substituted for Ser at position 25 of the V_L domain.

4. The antibody of claim 1, wherein the V_H domain and the V_L domain are separated by a linker.

5. The antibody of claim 1, wherein the V_H domain and the V_L domain are adjacent.

6. The antibody of claim 5, wherein the V_H domain and the V_L domain have a V_L-V_H orientation.

7. The antibody of claim 1, wherein the two amino acid substitutions are a Gln at position 6 of the V_H domain and a Lys at position 62 of the V_H domain.

8. The antibody of claim 1, wherein the two amino acid substitutions are a Gln at position 6 of the V_H domain and a Val at position 12 of the V_H domain.

9. The antibody of claim 1, wherein the antibody has a V_L sequence as set forth in SEQ ID NO:1 and a V_H sequence as set forth in SEQ ID NO:1 with the following amino acid substitutions: Gln replacing Glu at position 6 of the V_H domain; Val replacing Ser at position 12 of the V_H domain; and Lys replacing Asn at position 62 of the V_H domain.

10. The antibody of claim 9, wherein the V_H sequence further comprises an Ala replacing a Ser at position 25.

11. The antibody of claim 1 comprising an amino acid sequence of SEQ ID NO:2 or SEQ ID NO:3.

12. A stable humanized anti-CD22 antibody that has an LL2 binding specificity and comprises a V_H domain and a V_L domain, wherein the sequence of the V_H and V_L domains is at least 70% identical to SEQ ID NO:1 and comprises at least two of the following amino acid substitutions with respect to SEQ ID NO:1: Gln replacing Glu at position 6 of the V_H domain; Val replacing Ser at position 12 of the V_H domain; or Lys replacing Asn at position 62 of the V_H domain.

13. The antibody of claim 12, comprising all three of the substitutions.

14. The antibody of claim 12, wherein the two amino acid substitutions are a Gln at position 6 of the V_H domain and a Lys at position 62 of the V_H domain.

15. The antibody of claim 12, wherein the two amino acid substitutions are a Gln at position 6 of the V_H domain and a Val at position 12 of the V_H domain.

16. An isolated nucleic acid encoding a stable anti-CD22 antibody having a V_H domain and a V_L domain, wherein the sequence of the V_H and V_L domains are at least 90% identical to SEQ ID NO:1, and wherein the antibody comprises at least two of the following amino acid substitutions with respect to SEQ ID NO:1: Gln replacing Glu at position 6 of the V_H domain; Val replacing Ser at position 12 of the V_H domain; or Lys replacing Asn at position 62 of the V_H domain.

17. The isolated nucleic acid of claim 16, wherein the antibody encoded by the nucleic acid comprises all three of the substitutions.

18. The isolated nucleic acid of claim 16, wherein the antibody encoded by the nucleic acid further comprises an Ala substituted for a Ser at position 25 of the V_L domain.

19. The isolated nucleic acid of claim 16, wherein the V_H domain and the V_L are separated by a linker.

20. The isolated nucleic acid of claim 16, wherein the V_H domain and the V_L domain are adjacent.

21. The antibody of claim 20, wherein the V_H domain and the V_L domain have a V_L-V_H orientation.

22. The isolated nucleic acid of claim 16, wherein the antibody encoded by the nucleic acid comprises the two amino acid substitutions: a Gln at position 6 of the V_H domain and a Lys at position 62 of the V_H domain.

23. The isolated nucleic acid of claim 16, wherein the antibody encoded by the nucleic acid comprises the two amino acid substitutions: a Gln at position 6 of the V_H domain and a Val at position 12 of the V_H domain.

24. An isolated nucleic acid encoding a stable humanized anti-CD22 antibody that has an LL2 binding specificity and comprises a V_H domain and a V_L domain, wherein the sequence of the V_H and V_L domains is at least 70% identical to SEQ ID NO:1 and comprises at least two of the following amino acid substitutions with respect to SEQ ID NO:1: Gln replacing Glu at position 6 of the V_H domain; Val replacing Ser at position 12 of the V_H domain; or Lys replacing Asn at position 62 of the V_H domain.

25. The isolated nucleic acid of claim 24, wherein the antibody encoded by the nucleic acid comprises all three substitutions.

26. The isolated nucleic acid of claim 24, wherein the antibody encoded by the nucleic acid encodes the two amino acid substitutions: a Gln at position 6 of the V_H domain and a Lys at position 62 of the V_H domain.

27. The isolated nucleic acid of claim 24, wherein the antibody encoded by the nucleic acid encodes the two amino acid substitutions: a Gln at position 6 of the V_H domain and a Val at position 12 of the V_H domain.

28. An immunoconjugate comprising a stable anti-CD22 antibody as set forth in claim 1.

29. The immunoconjugate of claim 28, wherein the immunoconjugate is recombinantly produced.

30. The immunoconjugate of claim 28, wherein the antibody is linked to an effector molecule.

31. The immunoconjugate of claim 30, wherein the effector molecule is a detectable label.

32. The immunoconjugate of claim 30, wherein the effector molecule is a therapeutic moiety.

33. The immunoconjugate of claim 32, wherein the therapeutic moiety is a small molecule.

34. The immunoconjugate of claim 32, wherein the therapeutic moiety is an enzyme.

35. The immunoconjugate of claim 34, wherein the enzyme is an RNAse.

36. The immunoconjugate of claim 35, wherein the RNAse is angiogenin.

37. The immunoconjugate of claim 35, wherein the RNAse is RapLR1.

38. The immunoconjugate of claim 32, wherein the therapeutic moiety is a cytokine.

39. The immunoconjugate of claim 38, wherein the cytokine is IL-2.

40. The immunoconjugate of claim 38, wherein the cytokine is IL-6.

41. The immunoconjugate of claim 38, wherein the cytokine is IL-12.

42. The immunoconjugate of claim 32, wherein the therapeutic moiety is a toxin.

43. A nucleic acid encoding the immunoconjugate of claim 28.

44. An immunoconjugate comprising an antibody as set forth in claim 12.

45. The immunoconjugate of claim 44, wherein said immunoconjugate is recombinantly produced.

46. The immunoconjugate of claim 44, wherein the antibody is linked to an effector molecule.

47. The immunoconjugate of claim 44, wherein the effector molecule is a detectable label.

48. The immunoconjugate of claim 44, wherein the effector molecule is a therapeutic moiety.

49. A nucleic acid encoding an immunoconjugate of claim 44.

50. A method of inhibiting growth of a malignant B cell, said method comprising:

contacting the cell with a composition comprising an antibody according to claim 1 or claim 12.

51. The method of claim 50, wherein the cell is in a mammal.

52. The method of claim 51, wherein the mammal is a human.

53. The method of claim 50, wherein the malignant B cell is a lymphoma cell.

54. The method of claim 53, wherein the lymphoma is B cell non-Hodgkin's lymphoma.

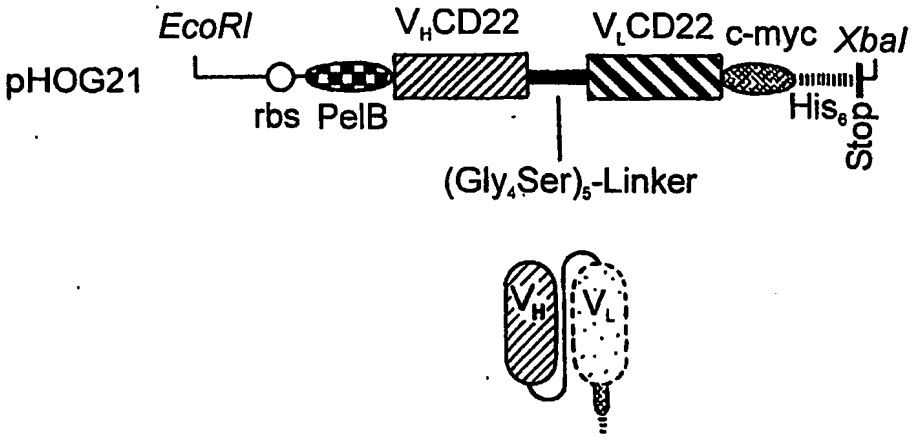
55. A method of detecting a cell that expresses CD22, said method comprising the steps of:

contacting the cell with a composition comprising an antibody of claim 1 or claim 12; and

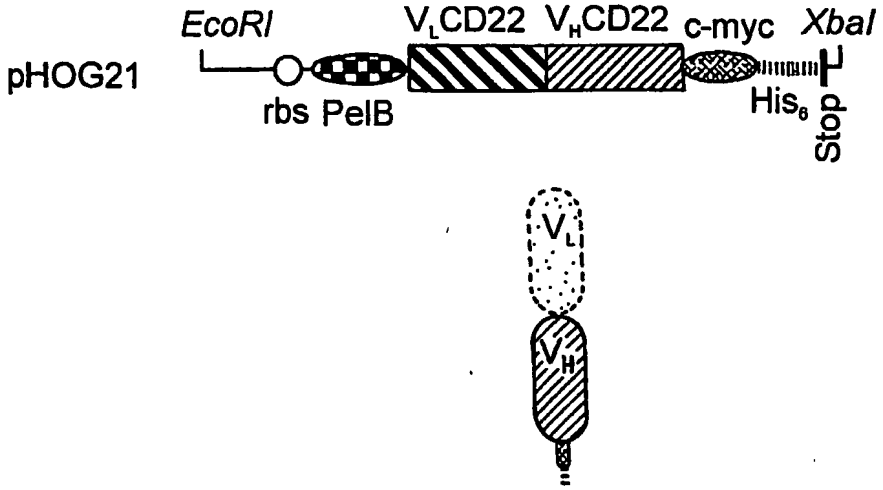
detecting binding of the antibody to the cell.

FIG. 1

(A) scFv with 15 amino acid GlySer-linker in V_H-V_L orientation



(B) scFv without linker (zero-linker construct) in V_L-V_H orientation



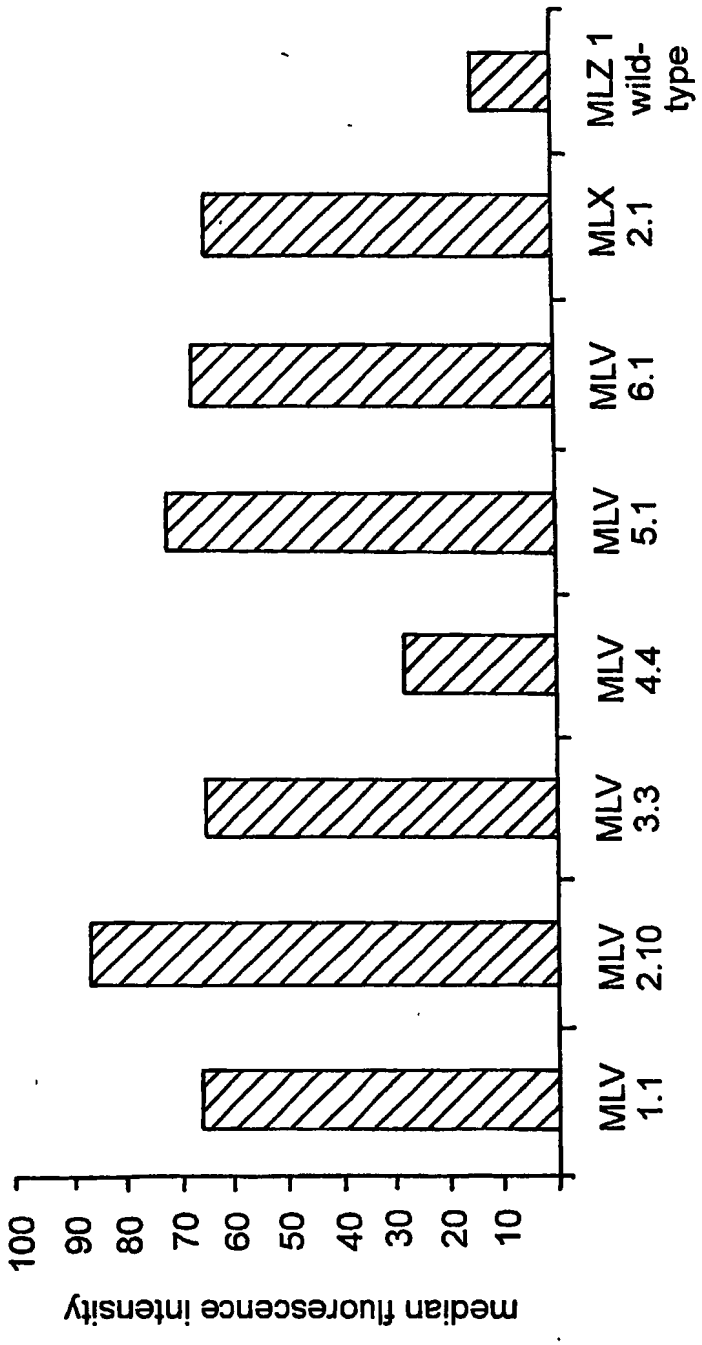
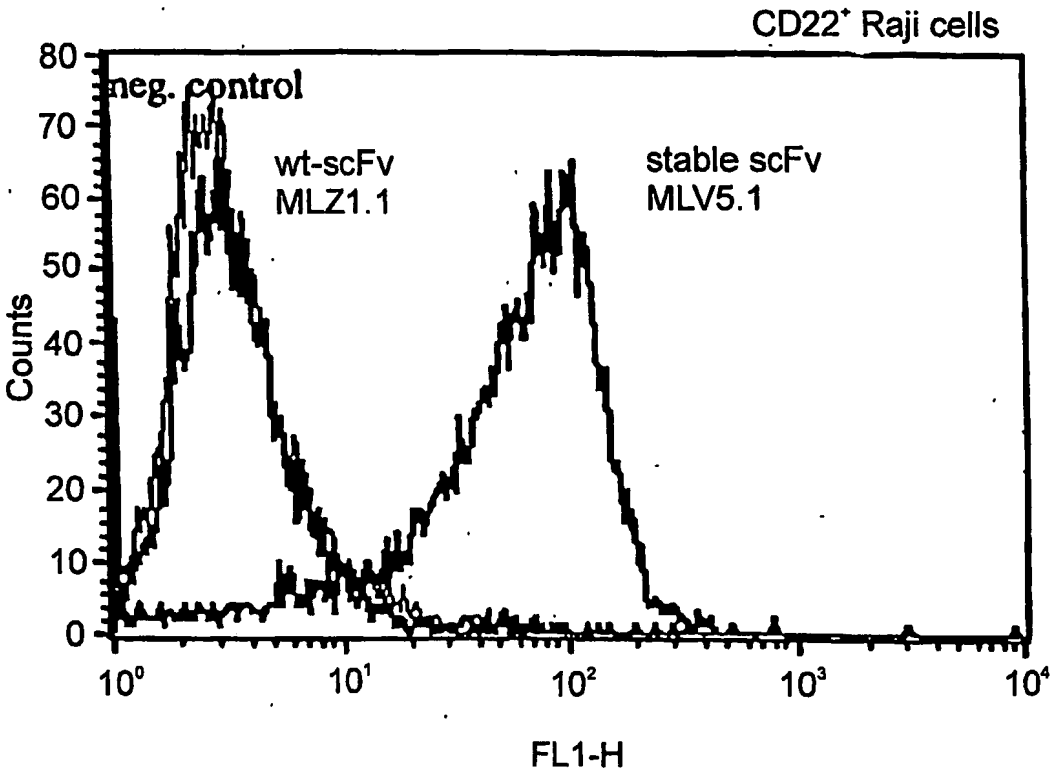

median fluorescence intensity
100
90
80
70
60
50
40
30
20
10
MLV 1.1
MLV 2.10
MLV 3.3
MLV 4.4
MLV 5.1
MLV 6.1
MLX 2.1
MLZ 1 wild-type
scFv variants

FIG. 2a

FIG. 2b


CD22+ Raji cells
neg. control
wt-scFv
MLZ1.1
stable scFv
MLV5.1
Counts
80
70
60
50
40
30
20
10
0
10^0
10^1
10^2
10^3
10^4
FL1-H

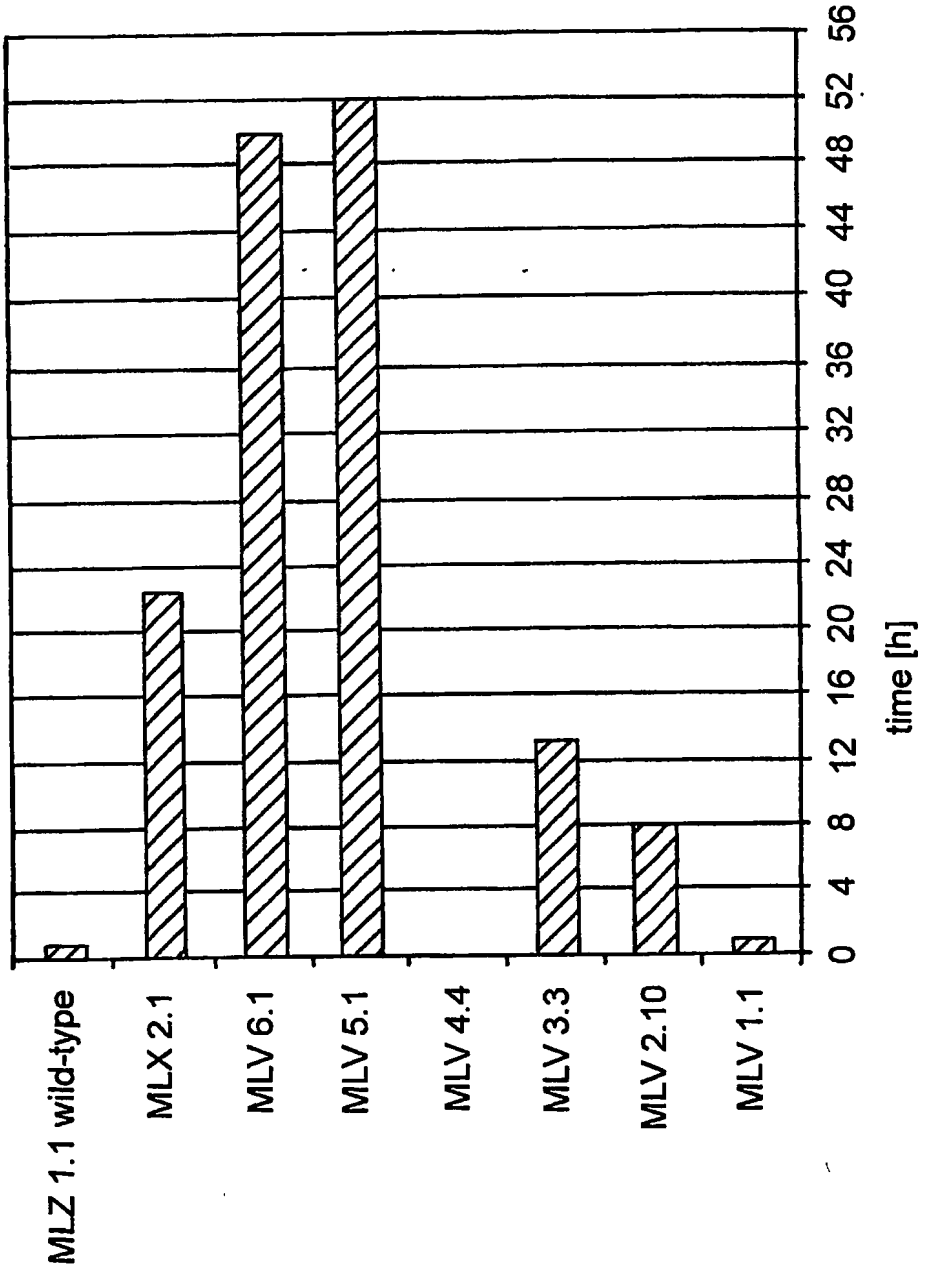
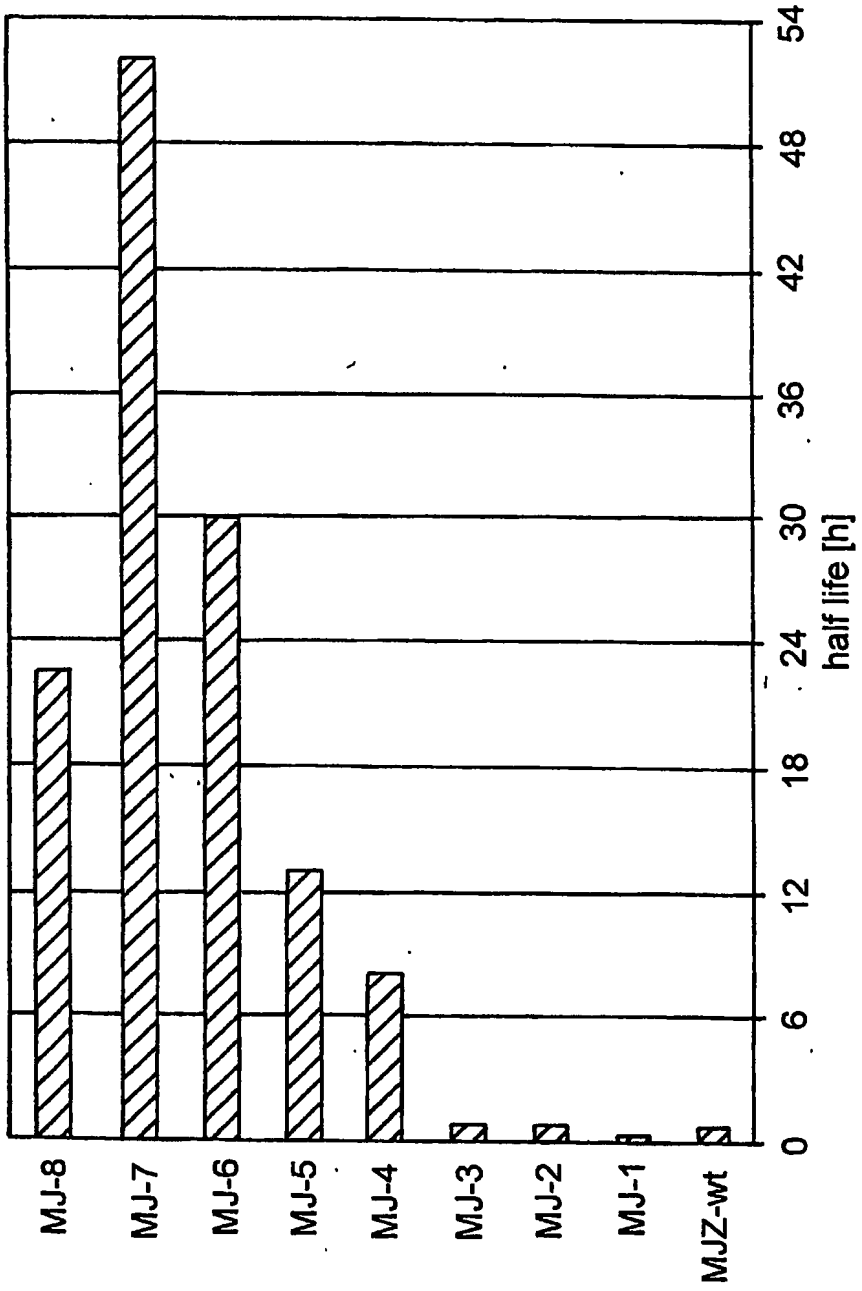

MLZ 1.1 wild-type
MLX 2.1
MLV 6.1
MLV 5.1
MLV 4.4
MLV 3.3
MLV 2.10
MLV 1.1
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56
time [h]

FIG. 3a


0
6
12
18
24
30
36
42
48
54
half life [h]
MJ-8
MJ-7
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MJ-5
MJ-4
MJ-3
MJ-2
MJ-1
MJZ-wt

FIG. 3b

FIG. 4

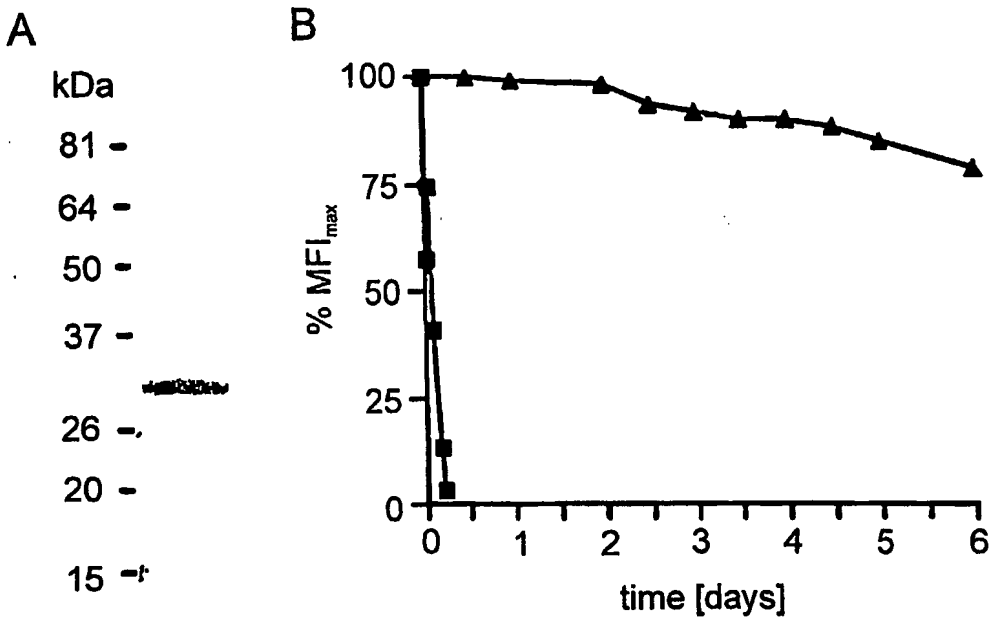

A
kDa
81
64
50
37
26
20
15
B
100
75
50
25
0
% MFI max
0
1
2
3
4
5
6
time [days]

FIG. 5a

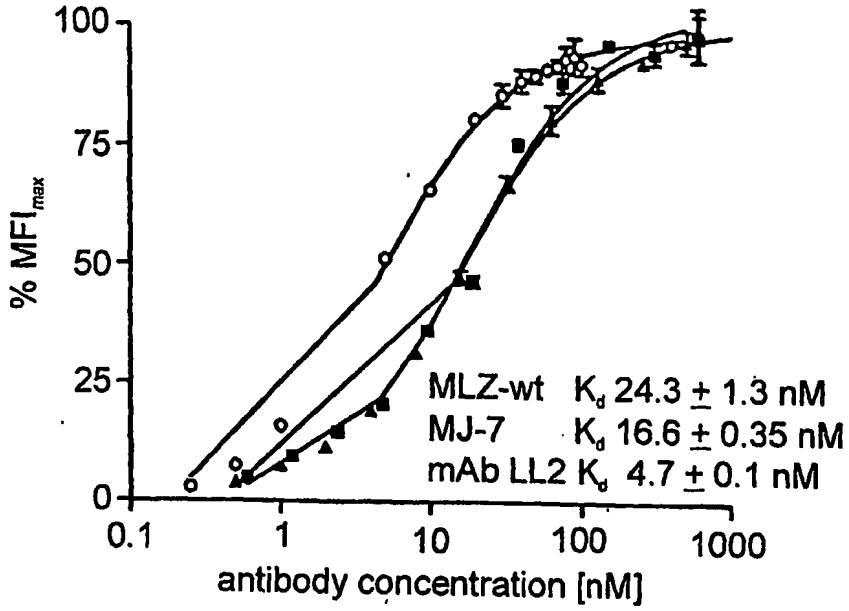
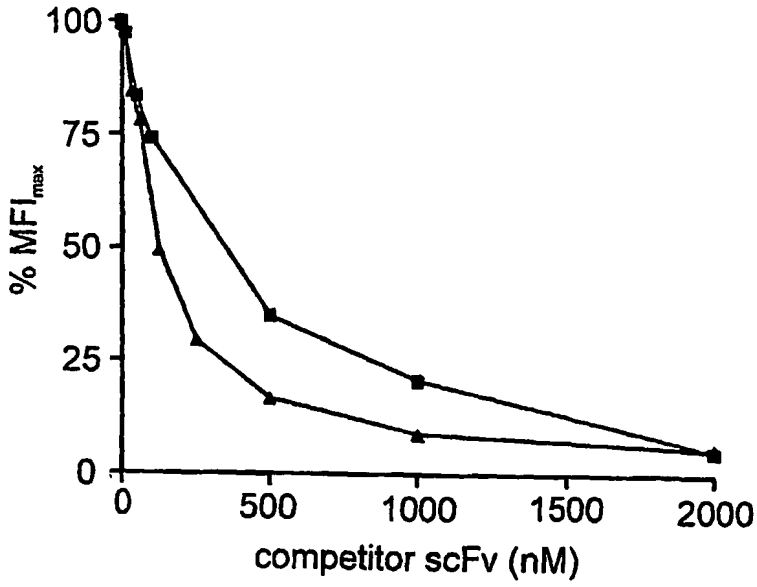

100
75
50
25
0
% MFI$_{max}$
0.1
1
10
100
1000
antibody concentration [nM]
MLZ-wt K_d 24.3 ± 1.3 nM
MJ-7 K_d 16.6 ± 0.35 nM
mAb LL2 K_d 4.7 ± 0.1 nM

FIG. 5b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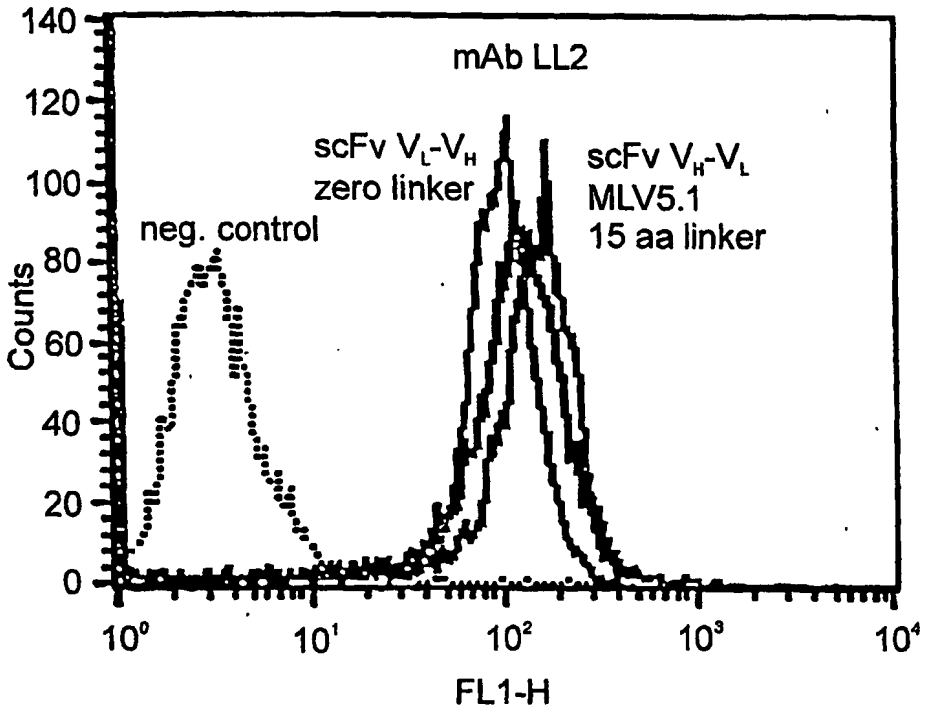
25
0
% MFI$_{max}$
0
500
1000
1500
2000
competitor scFv (nM)

FIG. 6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scFv V_L-V_H
zero linker
scFv V_H-V_L
MLV5.1
15 aa linker
neg. control
Counts
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120
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
FL1-H